STUDY OF BRAIN INJURY AND NEUROREGENERATIVE EFFECTS OF

D-CYCLOSERINE USING AN ASPHYXIAL CARDIAC ARREST RAT MODEL

By

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A

THESIS

Presented to the Faculty

of the University of Alaska, Fairbanks

in Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

By

Vélvá M. Combs, B.S.

Fairbanks, Alaska

May 2013

Abstract

Cardiac arrest (CA) affects over 300,000 Americans annually and results in severe brain injury (impaired motility, memory loss, or death) due to poor recovery. Over stimulation of glutamatergic N-methyl-D-aspartate receptor (NMDAR) channel activation, allows Ca^{2+} ions to enter cells, triggers a cascade of excitotoxic events and eventual neuronal death, but down-regulation of NMDAR post arrest may contribute to progressive injury. Previous studies have indicated that NMDARs are down-regulated within hours to days after resuscitation, therefore re-stimulation of NMDARs after CA should improve neurological outcome.

The purpose of this thesis was to: 1.) Implement an in vivo asphyxial CA (ACA) rat model at UAF to reproduce CA seen clinically in prenatal/pediatric populations, and 2.) Test the hypothesis that partial NMDAR agonist (D-cycloserine, DCS) would improve recovery from neuronal injury. Male Sprague Dawley rats (250-330g) were administered a low dose of DCS (10mg/kg, IP) 24 and 48hr after resuscitation from either 6 or 8-min ACA. Behavioral Neurological Deficit Scores (NDS) were taken at 2hr and daily for 7 days after ACA to assess injury. Histopathology assessed CA1 hippocampal neuronal injury. DCS had no effect on neurological improvement but the ACA model produced significant brain injury in rats regardless of CA duration.

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Acknowledgements

This thesis is dedicated in the loving memory to my mother, Lily DO'Villa, and my best friend, Stacy Arevalo, who both always believed in me even when I sometimes failed to believe in myself. I would also like to thank the following friends and family members who have heavily contributed to helping me see this thesis through to completion amongst major personal hardships: Barbara Knight, Raquel Silot, Angel Silot, Natania Silot, Jim Vaughn, May Gelera, Divine Smith, Karen Deras, Dizzeray Ai, Richard Allen, Sandra Figueroa, Brandi Fleshman, Zach Meyers, Kim Fackler, Michael Smith, Karina Gonzales, Ashley Berndt-Richie, Chad Richie, Eric Hulbert, Xiaodian Hulbert, Theresa Woldstad, Jonathan Manker, Kristine Adams, Kelly Kealy, Sophie Chowdhury, James Stone, and many others. Of course, special thanks goes to my committee (Dr. Kelly Drew, Dr. Abel Bult-Ito, Dr. Michael Harris) for taking the time out of their busy schedules to critically evaluate the merit of this thesis using their expertise to make it into something of important value. I would like to especially thank my advisor Kelly Drew for always supporting me and for being such a positive mentor to help me push forward when, at many times, I did not want to. I also would like to thank the Chemistry and Biochemistry Department Chair, Dr. William Simpson, for always being so positive and helpful with any questions or concerns I had.

I would like to personally thank Dr. June Sun, Heather Crispell, Dr. Tulasi Ram Jinka, Jasmine Davis, and Lindy Smith for surgical assistance, post-operative care monitoring, and moral support; Jeanette Moore, Zachary Carlson, and Joel Vonnahme for

blinding experimental drug treatments; Dr. Kelly L. Drew, Dr. June Sun, and Kimberly E. Iceman for initial LabScribe and iWorx equipment setup, data analysis, and troubleshooting; and finally Lori K. Bogren for additional technical assistance. I would also like to thank my colleagues at the Department of Neurology (D4-5), University of Miami Miller School of Medicine, Dr. Kunjan R. Dave, Dr. Miguel A. Perez-Pinzon, Isabel Saul, Ami P. Raval, Guillermo Fernandez, for further technical assistance and troubleshooting. I would also like to thank Dr. Julie McIntyre for her assistance with statistical analysis.

I would also like to thank the following funding agencies for providing financial support, in which, this study would have not otherwise existed without: National Institutes of Health (NIH) grant NS41069-06, US Army Medical Research and Materiel Command (# 05178001), UAF Center for Research Services, and Flint-Hills Resources. Lastly, thanks to all the animals whose lives were sacrificed for this study. Without their significant contribution, medical research would not be feasible and as successful as it is in contemporary society.

CHAPTER 1

General Introduction to Excitotoxic Effects Resulting From Cerebral Ischemia

1.1. Overview

This chapter will provide background on cerebral ischemia, cerebral ischemic subtypes (focal and global ischemia), and the excitotoxic mechanisms that occur during cerebral ischemic injury (e.g. membrane depolarization, hyperactivation of N-methyl-D-aspartate receptors [NMDAR], D-serine binding, etc.).

1.2. Cerebral Ischemia

Cerebral ischemia occurs in one of the following forms: focal ischemia (stroke), secondary injury ischemia following head trauma (*TBI, traumatic brain injury*), or as global cerebral ischemia (or cardiac arrest) (Rossi et al., 2007). Cerebral ischemia involves highly complex mechanisms where the brain's energy supply is compromised either focally (e.g. artery blockage in stroke) or globally (e.g. asphyxial cardiac arrest)—this results in severe brain damage which is the leading cause of death and long-term disability (Rossi et al., 2007). Focal ischemia during stroke is the most common form of ischemia and occurs when there is an abrupt disruption of blood flow to one or more specific areas of the brain.

Approximately 740,000 new cases of stroke occur annually in the U.S. (O'Neill and Clemens, 2001). Ischemic stroke can result in speech impairment, postictal seizure, facial weakness, headache, hemiparetic or ataxic gait, hypoglycemia, and general weakness in half of the body, such as in the arm or leg. Stroke can be classified as ischemic stroke, subarachnoid hemorrhage, or intracerebral hemorrhage and is usually diagnosed through use of neuroimaging (computed tomography, CT or magnetic resonance imaging, MRI) used to detect the lesions and other pathological processes involved and vascular areas affected (Yew and Cheng, 2009). Elevated intracranial pressure (ICP) and spontaneous intracerebral hemorrhage (ICH; accounting for 15-20% of all strokes) usually accompanies serious stroke injuries due to brain swelling from edema and results in poor prognosis or even fatality (Kimelberg, 1992, Hazell, 2007).

Traumatic Brain Injury (TBI) involves secondary ischemic injury and consists of complex pathophysiological processes caused by a wide range of plausible injury causes thus affecting TBI impact. Approximately 2% of the U.S. population will suffer long-term disability following TBI. TBI can occur either directly (cerebral injury) or indirectly (supratentorial trauma to the cerebrum) which can result in tremors, impaired fine motor and balance skills, ataxia, sensory deficits, etc. (Potts et al., 2009). Mortality increases even more in older patients when it can reach between 30-50%. Among people suffering TBI, 90% are expected to die within 48 hours of onset due to uncontrolled intracranial pressure that usually builds up and causes brain stem herniation and neuronal death (Park et al., 2008). Effective treatment for TBI remains a major problem

worldwide. The most effective treatments will require a multifactorial approach to prevent its rapid onset (Hazell, 2007).

Global ischemia resulting from cardiopulmonary arrest (CA) is the other form of ischemia. It remains one of the main causes of death in the U.S. even in light of fast emergency responses and improved defibrillation techniques. This type of ischemia affects the whole body including but not limited to the kidney, brain, and heart (Wang-Fischer, 2008). Cardiac arrest is defined as a sudden termination of blood supply and cardiac output and can be subdivided into two main categories: primary and secondary. Primary CA involves arrhythmias (ventricular fibrillation, VF, or ventricular tachycardia, VT) and is caused by a number of factors such as myocardial infarction, electrocution, or drug overdose. Secondary CA involves various causes such as respiratory arrest, hypothermia, airway obstruction, severe hemorrhaging, electrolyte imbalances, etc. (Robinson, 2004). Cardiac arrest (CA) affects over 300,000 people in the U.S. annually, in which only 70,000 are resuscitated and 42,000 die from the resulting brain injury (Krause et al., 1986). This injury results in either fatality or neurological impairment due to *global cerebral ischemia* (insufficient blood circulation to the entire brain). Neurological impairments include short-term memory loss, speech impediment, and impaired motor functions. Neurons continue to die from days to months after ischemia. Only 3-10% of CA survivors resume normal function, therefore there is greater need for further development of novel therapies to lessen ischemic effects post-injury in order to improve overall survival and outcome (Krause et al., 1986). The period of time occurring after CA injury and resuscitation (ROSC ["resumption/restoration of spontaneous

circulation"] or "CPR") is known as the *post-cardiac arrest syndrome*. This syndrome involves a myriad of pathophysiological processes including reperfusion brain injury, myocardial dysfunction, and systemic ischemia/reperfusion response, which cause the most damage to the brain and body (Neumar et al., 2008).

Potential CA treatments include drug treatments (the use of agonists and antagonist blockers/enhancers such as to limit excitotoxic cellular load or counteract its effects), ischemic preconditioning (which occurs before onset of subsequent injury), and therapeutic hypothermia (Neumar et al., 2008). However, there are barriers that prevent optimization and implementation of post-cardiac arrest care and treatment, including limited resources and/or requiring intensive care and monitoring; which are costly, and reliability and accuracy of early prognostication (<72hr after injury) remains limited and unclear (Neumar et al., 2008).

1.3. Excitotoxicity

N-methyl-D-aspartate receptor (NMDAR) excitotoxicity resulting from excessive extracellular glutamate release plays a key role in producing neuronal damage (Rothman and Olney, 1995). The glutamate hypothesis states that glutamate excitotoxicity leads to the development of several chronic neurodegenerative disorders (e.g. TBI, ischemia) caused by neuronal degeneration and dysfunction in response to CNS insult (Lau and Tymianski, 2010). During ischemia a cascade of excitotoxic events occur simultaneously within seconds after onset of injury, e.g., failed ATP ion pump function [Na^+/K^+

ATPase], membrane depolarization (efflux of extracellular glutamate; sharp increase of intracellular Na^+ and Ca^{2+}, and efflux of K^+ and Mg^{2+}) resulting in the opening of voltage-gated ion channels, and activation of a series of programmed cellular death pathways (Kristian and Siesjo, 1997). Astrocytes might also be involved in the release of ATP initiating the intercellular Ca^{2+} propagated waves in neighboring cells by activating purinergic (P2Y) receptors (Rossi et al., 2007).

Excessive glutamate release plays a key role in excitotoxic neuronal death following brain ischemia and trauma by triggering NMDAR activation. When glutamate binds to the glutamate-gated NMDAR (Carroll and Zukin, 2002) (with the endogenous cofactor glycine and/or D-Serine, D-ser, present), the channel is opened, leading to calcium influx into the cell and eventual decline in normal brain function. Ca^{2+} influx causes Ca^{2+} dependent production of ROS (reactive oxygen species) from disruption of the mitochondrial electron-transport chain, and release of 'free radicals' (Dykens, 1994). The time course of excitotoxic events can also differ between species such as rodents and humans. Previous microdialysis studies comparing TBI and stroke between humans and rats found that increases in glutamate only last a few minutes in rats but lasted anywhere from 6hr up to several days in humans (Biegon et al., 2004). This key difference could potentially play a vital role in determining how to treat excitotoxic ischemic effects in humans, especially when using rodent animal models to mimic human clinical neuropathologies.

1.4. NMDAR & D-serine

NMDARs are comprised of NR1 and NR2 subunits consisting of coagonist (glycine, and D-serine) and agonist (glutamate) binding sites necessary for receptor activation leading subsequently to 1.) Depolarization, 2.) Intracellular electric current, 3.) Ca^{2+} influx, 4.) Production of cGMP, 5.) Neurotransmitter release from vesicles, and after successive stimulation 6.) Neuron death (Nishikawa, 2005). NMDARs require both glycine and glutamate receptor activation and can be distinguished from similar kainite and AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptors by its need for simultaneous electrical and chemical stimulation through its ion channel (Furukawa and Gouaux, 2003). The NMDAR is unique among most ligand-gated ion channels since two different ligands must simultaneously be present on the receptor in order to conduct Na^{+} and Ca^{2+} ions.

Glutamate-gated NMDARs are densely localized in the hippocampus (particularly the CA1 region) and activation of these receptors plays an important role in formation of memory and learning (Biegon et al., 2002, Biegon et al., 2004). However, recent research indicates that NMDARs are also located on the myelin sheath of oligodendrocytes, glial cells producing the myelin sheath of axons in the brain; this is contrary to the belief that NMDARs were not involved in oligodendrocyte/myelin injury since they were not present here (Lipton, 2006). NMDARs are usually involved in synaptic plasticity and in a number of diseases such as schizophrenia (Furukawa and Gouaux, 2003).

Suppression of excitotoxic glutamate release, especially via inhibition of NMDAR activation using NMDAR antagonists, after ischemic insult can help reduce neuronal death and improve overall outcome (Biegon et al., 2004). NMDAR antagonists have been found to be effective in improving outcome in focal ischemic models, yet have little effect on improving outcome in global ischemic models (Lau and Tymianski, 2010). The problem with using NMDAR antagonists is that the effect is short-lived: antagonists only appear to be beneficial in a short time window given either before or immediately after injury and lose efficacy if given over 30 min to 1hr or greater post-injury (Biegon et al., 2004). Clinical trials of NMDAR antagonists developed to decrease brain injury have failed suggesting alternative types of treatment are necessary (Biegon et al., 2004). Alternative treatments involving the use of NMDAR agonists have been shown to be neuroprotective; especially since NMDARs have a dual function in which they can either produce neuronal death or survival via receptor subunit modulation (Kingston et al., 1999, Dos-Anjos et al., 2009). Liu et al., (2007) suggested neuroprotection after stroke insult is attributed to activation of NR2A-containing receptors. After 48hr post-injury, NR2A subunit expression in the hippocampus (an area highly vulnerable to ischemia) is reduced by half in comparison with NR1 and NR2B subunits which suggests that NR2A plays a critical role in regulating NMDAR expression (Dos-Anjos et al., 2009). This effect points to a functional deficit and desensitization ("down-regulation") of NMDARs rather than overstimulation as the main culprit for poor neuronal outcome following neuronal injury hours and days after injury, thus hinting at the need for alternative forms of treatment besides NMDAR antagonists (Biegon et al., 2004).

NMDAR coagonists can also provide neuroprotection. D-serine (D-ser), an endogenous NMDAR coagonist for NR1/NR2 subtypes; is densely concentrated throughout the brain, especially in higher brain function areas (Nishikawa, 2005). D-cycloserine (DCS) (a positive modulator and partial NMDAR agonist) binds to the same glycine site of NMDARs to which D-ser normally binds (Nishikawa, 2005) and plays an important role in recovery of cognitive function subsequent to brain injury (Temple and Hamm, 1996, Yaka et al., 2007). DCS binding to the glycine site is characteristically seen as one of many primary effects that speed up NMDARs from further desensitization and repeated and prolonged activation mediates physiological response (Temple and Hamm, 1996). DCS is already approved for antimicrobial use in humans and is considered to be a promising therapy for post-injury treatment of cognitive disorders like TBI (Temple and Hamm, 1996). Since DCS binds to the same site as endogenous D-serine, DCS could be used as a potential therapeutic treatment option to restore activation of down-regulated NMDARs and improve memory deficits resulting from cerebral ischemia.

1.5. Conclusion

This thesis will focus on the following topics: i.) Implementation of an in vivo asphyxial cardiac arrest (ACA) rat model at the University of Alaska, Fairbanks (UAF) to reproduce cardiac arrest (CA) and assess brain injury resulting from global cerebral ischemia pathology seen clinically in both perinatal and pediatric populations, and ii.)

Test the hypothesis that a partial NMDAR drug agonist (D-cycloserine, DCS) would improve recovery from neuronal injury.

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CHAPTER 2

Health Implications and Technique Assessment of An Asphyxial Cardiac Arrest (ACA) Rat Model Transferred to The University of Alaska, Fairbanks (UAF) From The University of Miami Miller School of Medicine

2.1. Abstract

Cardiac arrest (CA) results in severe neurological impairments and death through excitotoxicity. Established animal models such as the four-vessel occlusion (4VO) and two-vessel occlusion (2VO) with hypotension have been used to study the pathology associated with CA. The asphyxial cardiac arrest (ACA) rat model has previously been established to reproduce reliable post-CA neurological injury and true global ischemia seen clinically in perinatal and pediatric populations. The ACA model includes surgical and post-operative care components allowing for long-term monitoring of neurological degradation compared to 2VO and 4VO animal models. The ACA model is easier to implement and produces whole brain ischemia but can also be and time-consuming and result in low successful resuscitation rates with respect to surgical skill level.

2.2. Asphyxiation Causes Cardiac Arrest

Asphyxiation is defined as oxygen deprivation caused by impaired respiration in which gas exchange required for normal cellular metabolism is disrupted (Elsner, 1983). Asphyxiation can lead to severe *hypoxia* (inadequate oxygenation supplied to specific tissues such as the lungs, heart, or brain) and often results in cardiac arrest, with subsequent global cerebral ischemia and brain damage. *Asphyxial Cardiac Arrest* (ACA) is clinically defined as the termination of mechanical activity by the heart caused by insufficient respiration (asphyxiation), lack of responsiveness, and the inability to generate a central pulse (Goldstein, 1982, Myerburg et al., 1982, Greene et al., 1989, Zaritsky et al., 1995, Manole et al., 2008) and results in hypoxic brain injury (Moulaert et al., 2010). Approximately 50% of all CA patients are successfully resuscitated with 10-20% of those patients making good recovery; most suffer severe disability (usually resulting in expensive ICU hospital costs sometimes up to $95,000 for comatose survivors (Gray et al., 1991)) or death (Geraghty and Torbey, 2006). Some general causes of asphyxial cardiac arrest include but are not limited to the following factors: severe acute respiratory failure, obstruction of airway passages (e.g. foreign objects, tumors, etc.), acute asthma attacks, pneumonia, asphyxiation by hanging (suicide), and acute intoxication (Lah et al., 2011); other asphyxiation causes also include near-drowning, near-SIDS (sudden infant syndrome), hypoxemia (low blood oxygen), choking, and extrapyramidal side effects from drug use (e.g. antipsychotics & dyskinesia) (Shannon and Kelly, 1982a, b, Macnab, 1995, Safar et. al., 1996, Robinson, 2004).

This chapter will focus on several factors regarding ACA: incidence in different human populations (perinatal, pediatric, and adult), current treatment, previous animal models used to mimic ACA pathophysiology, and extensive detail regarding the ACA rat model (equipment, surgical procedure, and trouble-shooting). The ACA rat model produces CA and brain injury that closely matches pathophysiology seen clinically and potentially offers the best model option for testing future therapies.

2.3. Perinatal (Neonatal) Asphyxiation

Permanent neurological brain injuries such as mental retardation, blindness, deafness, cerebral palsy, epilepsy, and various learning disabilities (Robertson et al., 2009) seen in children are commonly caused by hypoxia and ischemia during perinatal age (third gestation trimester through one month postnatal) (Scher, 2001, Berg et al., 2008a, Robertson et al., 2009). Cerebral palsy (CP) usually results from either asphyxiation or some other pathology suffered during the antepartum (pre-labor) time period; <20% of CP patients suffered brain injury exclusively from intrapartum (during labor) asphyxiation (Scher, 2001). Incidences of asphyxial injury in infants occur at a rate of 0.2 to 0.4% of all full-term births and at even higher rates for low birth weight and preterm infants (Robertson et al., 2009). Asphyxia can also occur in brief stints during the delivery portion in all births resulting from various reasons such as inadequate oxygenation in arterial blood caused from compression of the umbilical cord or hypovolemic/hypotension heart failure compounded by respiration retraction (Elsner,

1983, Morley, 2005). Respiration can be depressed in new-born infants by central mechanisms such as hypoxia, and reflexively through stimulation of upper-airway receptors, and initial bradycardia during apnoeic episodes due to decreased pulmonary stretch receptor activity and loss of normal rhythmic central respiratory drive (Elsner, 1983). Treatment against this effect should incorporate reversal of the hypoxic and apnoeic state by restoring adequate gas exchange (Elsner, 1983).

Intrapartum asphyxia and cerebral perfusion results in hypoxic ischemic-encephalopathy (HIE). HIE occurs approximately one in 12,500 live births with a steady decline over recent years, while the global CP rate is estimated to be approximately 1 to 2 cases per 1,000 live births (Phelan et al., 2005). Neurological brain damage in neonates can be due to the presence or sometimes the absence of severe acidosis (e.g. umbilical artery $pH < 7$, and a base deficit ≥ 12) which can occur at the time of birth and result in hypoxia and slower heart rate (<100 bpm) (Phelan et al., 2005). Fetuses try to counteract oxygen deprivation effects by diverting blood from less vital organs (kidney or liver) to more vital organs (brain, heart, and adrenal glands) speeding up red blood cell production and improving overall fetal oxygenation (Phelan et al., 2005).

2.4. Pediatric Asphyxiation

Nearly 60% of all CA etiology seen in children is marked by an asphyxiation period occurring prior to loss of circulation (Manole et al., 2008, Robertson et al., 2009).

Pediatric CA is typically caused from circulatory shock, respiratory failure, or a combination of both. Arrhythmogenic ventricular fibrillation (VF) arrests are seen more commonly in adults rather than in children (Nadkarni et al., 2006, Neumar et al., 2008). VF/VT (ventricular tachycardia) arrests occur in ≈ about 10% of in-hospital CA and 5-20% of out-of-hospital pediatric CA (Neumar et al., 2008). In-hospital pediatric CA occurs in 2-6% of all children admitted to pediatric intensive care units (PICUs) (Kuisma et al., 1995, Slonim et al., 1997, Suominen et al., 2000, Berg et al., 2008a) and in 4-6% of children after cardiac surgery (Rhodes et al., 1999, Parra et al., 2000, Berg et al., 2008b). Out-of-hospital cardiac arrests (OHCAs) occur in approximately 8-20/100,000 children annually (Young et al., 2004, Donoghue et al., 2005, Berg et al., 2008a), with a 100-fold higher incidence for in-hospital cardiac arrests (IHCAs) (Morris and Nadkarni, 2003, Berg et al., 2008a). About two-thirds of IHCA patients are successfully resuscitated (Parra et al., 2000, Reis et al., 2002, Meaney et al., 2006, Nadkarni et al., 2006, Samson et al., 2006) with over 25% survival to hospital release (Berg et al., 2008a).

The most common causes of CAs are asphyxiation due to acute hypoxia or hypercarbia, ischemia from inadequate blood flow to the heart (e.g. hypovolemic shock or myocardial dysfunction), and arrhythmias stemming from ventricular fibrillation, VF, or ventricular tachycardia, VT (Berg et al., 2008b). Asphyxial pediatric CA is usually caused by hypoxemia, hypercarbia, acidosis, and hypotension. Respiratory compromise is the most common cause of non-traumatic pediatric CA and can be secondary as well resulting in upper or lower airway constriction and/or obstruction leading to secretions,

aspiration, infection, suffocations, trauma, and/or depressed respiratory drive secondary to neurological impairment (Manole et al., 2008).

Drowning is a common cause of death where approximately 8,000 deaths occur annually in the U.S., caused either by asphyxiation alone or in combination with water intake (Elsner, 1983). Near-drowning is the third most common cause of death with a majority of victims under the age of 20 years. These drowning victims suffer anoxic encephalopathy, acute respiratory failure (ARF), or hypoxemia (defined as having Po_2 60 mmHg without supplemental O_2) (Gregorakos et al., 2009). Drowning pathophysiology is caused by water aspiration into the lungs which leads to a multitude of fluctuating sympathetic and parasympathetic over-activity: blood pressure first increases followed by a gradual decline; excessive glucose, hypoxia and CO_2 accumulate due to acidosis; and eventually circulatory failure occurs (Golden et al., 1997, Vaagenes et al., 1997). Near-drowning can also be paradoxically neuroprotective for children. The surface area to mass ratio and small body mass of young children in combination with immersion into cold water (hypothermia protection), improves survival due to the absence of higher brain (cortical) function and loss of voluntary muscle movement; especially if they are knocked unconscious prior to water submersion (Elsner, 1983).

Another cause of pediatric asphyxiation is Sudden Infant Death Syndrome (SIDS). SIDS is the third leading cause of infant mortality (~2300 infant deaths) in the United States annually and occurs in infants of 1 month to 1 year in age and of male gender) (Moon et al., 2007, Moon and Fu, 2012). Causes of SIDS range from various

factors such as environmental, genetic, smoking exposure (usually during pregnancy), low birth weight, overheating, bedding material, negligence, sleeping position, and ethnicity (twice as many mortality rates [death/live births] for Alaska Native/American Indian infants [112/100,000] and African American infants [99/100,000] compared to Caucasian infants [55/100,000]) (Moon et al., 2007, Moon and Fu, 2012).

2.5. Adult Asphyxiation

Adult asphyxial CA can occur from various internal and external causes of either cardiac or non-cardiac etiology such as: non-traumatic bleeding, pulmonary embolism, intracranial processes, pneumonia, asthma, convulsions, malignancy, hemorrhagic pancreatitis, intoxication, trauma, near-drowning, choking, hanging [suicide], and carbon monoxide intoxication (Kuisma and Alaspaa, 1997). Ventricular fibrillations tend to be the main cause of adult CA versus asphyxia for pediatric/perinatal CA (Katz et al., 1995, Kochanek et al., 2009). Outcome from CA results in a reduction in the quality of life marked by cognitive impairments, fatigue, memory problems, post-traumatic stress, depression, and anxiety (Moulaert et al., 2010). In terms of drowning asphyxiation, survival rate was higher in drowning victims than for out-of-hospital primary cardiac arrest (OHPCA) patients (Grmec et al., 2009). In an cardiac arrest drowning study conducted from Feb. 1998 to Feb. 2007 in the town of Maribor (located in the European territory, Slovenia), 67% (528/788) cardiac arrests with resuscitation were OHPCA, while 4% (32/788) cardiac arrests with resuscitation were of drowning victims (Grmec et

al., 2009). The survival rate among the drowning patients was significantly higher, 44%, compared to OHPCA patients at 22% (Grmec et al., 2009).

Adult cardiac arrest usually occurs as a phenomenon called "Sudden cardiac arrest". The onset of sudden cardiac arrest (SCA) usually has no real onset of symptoms prior to injury. Out-of-hospital cardiac arrests (OHCAs) are comprised of trauma, intoxication, near-drowning, non-traumatic bleeding, and pulmonary embolism.

Thirty percent of OHCAs are non-cardiac in origin and most SCAs happen to also tend to be non-cardiac in origin. The incidence of sudden OHCA of non-cardiac origin was 26/100,000 patients annually while trauma cases made up 20/100,000 patients annually, with an average age of about 49 years old (≈65% male; 4% under 16 years old) (Kuisma and Alaspaa, 1997).

Some plausible causes of SCA in adults include channelopathies (e.g. long QT syndromes and molecular anomalies [e.g. type 2 ryanodine receptor mutation]), genetic predisposition, coronary artery atherosclerosis which has accounted for 40% and 65% of major deaths in females and males respectively (coronary disease resulting in death was even seen in 15-49 year olds), lethal arrhythmia (ventricular fibrillation), acute coronary insufficiency in absence of coronary artery atherosclerosis (e.g. fibromuscular dysplasia, coronary artery narrowing), respiratory failure (e.g. sleep apnea, asthma), exercise exertion (e.g. anomalies found in coronary or pulmonary arteries), myocardial ischemia (myocarditis in young adults), side effects from psychotropic medication (typical and atypical antipsychotic drugs), anaphylaxis, viral infections, subarachnoid hemorrhage, esophageal/gastrointestinal causes of sudden death, etc. (Langlois, 2009).

2.6. Post-Cardiac Arrest Syndrome And Brain Injury

The *post-cardiac arrest (CA) syndrome* characterizes the body's response to whole-body ischemic/reperfusion effects occurring after successful cardiopulmonary resuscitation (CPR) due to cardiac arrest injury (Negovsky, 1972, Neumar et al., 2008). Post-CA brain injury results in eventual neuronal degradation hours to days after CA due to lack of oxygen supplied to the brain (Neumar et al., 2008). Clinical manifestations include hypotension, seizures, memory impairment, hypoxemia, a comatose state, myoclonus, impaired cerebrovascular autoregulation, and brain edema (Krumholz et al., 1988, Pusswald et al., 2000, van Alem et al., 2004, Neumar et al., 2008). The brain's vulnerability to ischemia involves complex excitotoxic mechanisms including generation of free radicals, disruption of Ca^{2+} homeostasis, pathological protease cascades, and programmed apoptosis (Lipton, 1999, Neumar, 2000, Bano and Nicotera, 2007). Children are particularly vulnerable to post-CA brain injury due to their poor survival outcome (Manole et al., 2008). Therefore, implementation of anti-excitotoxic therapies should be pursued as potentially beneficial treatment options in order to preserve cerebral metabolic demands.

2.7. Treatment

Treatment against asphyxia can be simply avoidance and prevention against things that may cause asphyxia in the first place or implementation of therapies aimed at

restoring adequate cerebral blood flow either during or after CPR or ischemic insult if CA ensues. One such treatment is resuscitating newborns with room air rather than 100% oxygen to improve neurological outcome after CA since reactive oxygen and nitrogen species potentially worsen the outcomes of some neurological damage in animal models of CA (Manole et al., 2008). Another treatment option involves the use of neuroprotective drug therapies such as N-methyl-D-aspartate (NMDA)/α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/MK-801/Phencyclidine (PCP) antagonists, magnesium sulfate, and -aminobutyric (GABA) agonists (Aarts and Tymianski, 2005, Manole et al., 2008, Neumar et al., 2008).

Mild hypothermia has proven to be the most effective therapy against post-cardiac arrest injury (Hachimi-Idrissi et al., 2001, Hypothermia after Cardiac Arrest Study, 2002). Therapeutic hypothermia cools body temperature to between 32-34°C for 12-24 hours to increase energy stores required by the brain (Nilsson et al., 1975). Oxygen consumption during hypothermia is reduced to approximately 5% per °C between 22-37°C (Yenari et al., 2008).

The benefits of hypothermia treatment have been observed in near-drowning victims. People who have ceased vital function from drowning were recovered as long as 40 min. from their initial submersion. It has also been reported that the heart can be restarted after two hours of water submersion (Harries, 1986). Deep hypothermia (<33°C) has been shown, however, to cause such complications as bleeding, arrhythmia, sepsis, and even thrombosis but this effect has not been seen in infants with mild hypothermia

treatment (Hoehn et al., 2008). Hypothermia treatment for asphyxia newborns with encephalopathy did not improve survival or the condition of severely disabled infants but did have a significant impact on moderately affected infants (Hoehn et al., 2008).

In summary asphyxiation is a cause for cardiac arrest, especially in pediatric populations.

2.8. Animal Models of Global Cerebral Ischemia

Cardiac arrest leads to disability and death in part due to brain injury caused by global cerebral ischemia that ensues when cardiac function ceases. Several models have been developed to study global cerebral ischemia in animals. All of these models are limited by cardiovascular variables that differ from the clinical scenarios seen in humans.

2.8.1. Four-Vessel Occlusion (4VO)

The four-vessel occlusion (4VO) rat model is a model that reproduces global cerebral ischemia such as occurs during cardiac arrest in clinical situations by inducing transient forebrain ischemia. In this two-day procedure, permanent coagulation of the two vertebral arteries occurs first and then occlusion (via temporary ligation) of the two common carotid arteries occurs, allowing the hindbrain to still be partially perfused (Schmidt-Kastner et al., 1989). This effect allows for delayed cell death and reliable

outcome (Schmidt-Kastner et al., 1989). The 4VO rat model is relatively inexpensive and useful for studying the unusual delayed cell death pattern after injury onset and for testing drugs for neuroprotection; however, there are also limitations with this model including chance of improper cauterization technique or rupture of vertebral arteries or insufficient blockage of carotid arteries resulting in excessive bleeding, spinal cord damage, severe neurological deficits prior to 4VO, loss of righting reflex, or even death (O'Neill and Clemens, 2001). For example, there is a chance of potential error when locating vertebral arteries for permanent occlusion using electrocauterization and/or possible tissue damage resulting from excessive heat use or muscle trauma leading to complications and fatality during surgery (Small and Buchan, 2000).

2.8.2. Two-Vessel Occlusion (2VO) With Hypotension

Two-vessel occlusion (2VO) with hypotension rat model (along with 4VO model) is one of the most frequently used for research into molecular mechanisms of global neuronal damage (Ginsberg and Busto, 1989, Wang-Fischer, 2008). 2VO produces reversible forebrain ischemia by employing bilateral common carotid artery occlusion combined with systemic hypotension (50 mmHg). Two main histopathological changes occur in this model: 1.) Selective neuronal variability (e.g. pyramidal neurons of the hippocampal cornu ammonus (CA) subregion [curved, U-shape lamina field of the hippocampus (Gondi et al., 2010)] and 2.) Delayed neuronal death (necrosis occurs 2-3 days after reperfusion) (Raval et al., 2009). Reproducibility of ischemic damage is

greater than 90% and suitable for many research applications (neuroprotective agents, biochemical and molecular studies, etc.) (Raval et al., 2009). Although the 2VO with hypotension model is surgically easier to implement than the 4VO and allows for relatively easy monitoring of physiological variables, it can also produce overall variability in outcome (specifically, histopathology) which can result in profound variability beyond the experimenter's control due to the need to induce hypotension (Small and Buchan, 2000).

2.8.3. Asphyxial Cardiac Arrest (ACA) Rat Model

The asphyxial cardiac arrest model (ACA) produces systemic hypoxia-ischemia, arterial hypotension, and circulatory arrest in tissues negatively impacting the entire body (Dave et al., 2009). The ACA model best approximates a true clinical scenario that leads to global cerebral ischemia. The ACA rat model is an already established model for neurological injury after cardiac arrest, reproducing many neurological deficits in humans (Callaway and Logue 2009). This model also adds additional variables to the CA pathology, which include but are not limited to: hypercapnia (excessive CO_2 levels unable to expire out of the body), acidosis (lower pH in tissues), blood clots, lag in O_2 delivery from endotracheal intubation setbacks, etc. Some advantages to using this in vivo ACA model is that it can reproduce similar hypoxic and/or anoxic conditions that naturally occur in various brain diseases in humans and hopefully provide accessible ways to develop novel treatments by isolating specific mechanisms characteristic of this

ischemic injury (Matsuoka et al., 1997). Another benefit of using the ACA model is that the biochemical pathways associated with ischemia can be studied within seconds or minutes after onset mimicking real time ischemia that occurs clinically; drug efficacy can also be assessed based on the knowledge of these pathways (Dave et al., 2009). Using small animals such as rodents in this model proves to be more cost effective over using larger animals and produces clear, consistent and reproducible injury in the hippocampus and posterior caudoputamen with 8-10 min duration of asphyxia (Traystman, 2003). Global cerebral ischemia models provide a more reliable way of quantifying and assessing neuronal damage and possible treatment from neuroprotective agents since the amount of variability in induction is significantly reduced especially in comparison with focal ischemia models (Small and Buchan, 2000). Advantages and disadvantages of this model are summarized in **Table 2.1**.

2.9. ACA Surgical Procedure

This section will look at all aspects involved with the asphyxial cardiac arrest rodent model, including step-by-step analysis of how to implement mechanical ventilation via endotracheal intubation, venous cannulation and IV (intravenous) paralytic administration, asphyxial cardiac arrest induction, resuscitation technique, and post-operative care. Asphyxial cardiac arrest is achieved in mechanically ventilated animals. Paralysis is induced and mechanical ventilation is halted to achieve asphyxia. Asphyxia leads to a reproducible drop in both blood pressure and heart rate sufficient to produce

global cerebral ischemia. Animals are then resuscitated by rapid manual chest compressions and immediate delivery of epinephrine followed by sodium bicarbonate to adjust pH after resuscitation. The most difficult aspects of the procedure involve intubation and mechanical compression that can lead to inflammation and/or obstruction of the trachea and pulmonary edema.

2.9.1. Induction & Intubation

First, open pressure gauge pack and set up pressure transducer. Next, prepare injectables (see **Appendix D**, "Asphyxial Cardiac Arrest Setup") and fill two Tygon cannulae with heparinized-saline (hep-saline) using a 1cc syringe and 26ga luer stub adaptor. Fill one 10cc syringe with sterile saline. Leave sterile ends inside gas sterilized pack. Anesthetize with 5% isoflurane and a 30:70 mixture of O_2 (400-500mL/min) and N_2O (1L/min) via scavenger fluovac mask. This balance of N_2O ensures stable physiological levels for Po_2, Pco_2 and pH (Takeuchi et al., 1992).

Intubation procedure: Open sterile intubation pack**.** Place rat in a supine position while on rodent intubation stand (Hallowell EMC, Pittsfield, MA; Rodent Tilting Work Stand, Item No. 000A3467, $484.21; www.hallowell.com) with teeth secured by incisor loop strap (Hallowell EMC, Pittsfield, MA; Incisor Loops, 5 pk, Item No. 210A3490, $30.31; www.hallowell.com) sliding bars (optional) located on stand (animal will be off anesthesia mask during this procedure). Insert otoscope, with speculum (Henry Schein,

Inc., Melville, NY; Welch-Allyn Otoscope Pneumatic w/specula, 3.5V [20200]; No. 5662532, $186.99; Welch-Allyn Handle Rechargeable 3.5V [71000-A], No. 5662474, $154.49; Welch-Allyn Battery Rechargeable Orange 3.5V [72300]; No. 5662828, $45.79) attached and light turned on (Hallowell EMC, Pittsfield, MA; Rodent Tilting Work Stand, Item No. 000A3467, $484.21; Rat Autoclaveable Specula, Item No. 200A3588, $38.59, www.hallowell.com), against the palate of the mouth (with the curved slide facing downward) while holding and deflecting the tongue to the side with your free hand.

Next, place a drop of lidocaine gel (Lidocaine Hydrochloride Jelly USP, 2% R_x; 30 mL, NDC 17478-711-30 or equivalent) on the tip of the lidocaine applicator (Hallowell EMC, Lidocaine Applicator, Item No. 200A3590, $24.26, www.hallowell.com) and apply it to the epiglottis (arch shaped and located just above the vocal cords) while still holding the otoscope and speculum against the palate of the animal's mouth with the opposite hand. Lidocaine gel application should reduce epiglottis spasm as well as irritation in this sensitive region. Put animal back on anesthesia mask (rats 2-2.5% Isoflurane) for 2 min. Next, place a drop of lidocaine gel (Lidocaine Hydrochloride Jelly USP, 2% R_x; 30 mL, NDC 17478-711-30 or equivalent) on the tip of the lidocaine applicator (Hallowell EMC, Lidocaine Applicator, Item No. 200A3590, $24.26, www.hallowell.com) and apply it to the epiglottis (arch shaped and located just above the vocal cords) while still holding the otoscope and speculum against the palate of the animal's mouth with the opposite hand. Lidocaine gel application

should reduce epiglottis spasm as well as irritation in this sensitive region. Put animal back on anesthesia mask (rats 2-2.5% Isoflurane) for 2 min.

Next, take animal off mask and reinsert otoscope/speculum unit as described previously. This time use the endotrachael catheter (14ga 1.75in; BD Insyte™ Autoguard™, Shielded IV Catheter, REF# 381467; guided with a bent, custom blunt biomedical 17ga x 4in. needle, 17TWx3, Popper & Sons, New Hyden Park, NY #7427; blunt end can be made with a sanding tool) and insert it (with curved side facing up) into the trachea located via the opening between the two vocal cords. Movement of the vocal cords should be slowed down enough in order to facilitate this technique. Once the catheter is fully inside the trachea (only the orange hub should be sticking out of the mouth), quickly check if the catheter is truly in the trachea and not in the esophagus by holding a stainless steel instrument in front of the open end of the catheter (orange hub) so that condensation of respiratory moisture can be seen. If no moisture is visible, then it means that the catheter is in the esophagus. Repeat the aforementioned steps in order to reinsert endotracheal catheter into trachea. Discontinue surgery if more than two tries are needed to execute intubation. Return animal to home cage for at least 1 week before repeat attempt.

If an otoscope and endotracheal stand is unavailable for use, the trachea may be visualized to facilitate intubation. Clip the hair from the ventral neck and using a #10 or #15 blade or curved, blunt scissors make a 1-2cm incision along the ventral midline of the neck to expose the trachea and larynx. To prevent laryngospasm during oral

intubation swab lidocaine onto laryngeal folds with a small cotton tip swab moistened with 2 drops of lidocaine. (Lidoject; lidocaine 2% injectable, Butler Animal Health Supply, Dublin, OH 43107). Next, suture catheter to lip to avoid dislodging catheter during resuscitation.

To secure the catheter to the trachea, use a 3-0 silk suture (100 yds., waxed, USP 3/0 black braided silk suture, Lot No. 6253) about 4 inches in length and place through slit opening of a cutting needle (World' Precision Instruments, Inc.; cutting needle, Sarasota, FL; 5/16 Circle, 18mm cutting edge, spring eye, No. 501960, Lot No. 1080297400; www.wpiinc.com). Place cutting suture with silk suture thread underneath the last round rung of the orange hub unit of the endotracheal catheter while it is placed inside the intubated animal (whom is connected to the ventilator at this point). With the only the orange hub sticking out of the animal's mouth, make one throw around the last rung of the hub (so that the suture is secured to the catheter), lift up a small portion of the animal's lip with forceps and pass the cutting suture through the lip (toward surgeon), make one throw (away from surgeon), grab the rest of the silk thread and pull it through in order to make a knot (make two knots total). Be sure not to tie too tight to the lip so that it is easier to remove later on and doesn't damage the animal's lip.

For a terminal, non-recovery procedure, a tracheotomy may be substituted for oral intubation. The trachea will be exposed and a small hole will be made between the cartilaginous rings in the trachea and the 14ga 1.75in; BD Insyte catheter will be inserted in that opening in the trachea.

Shave throat (if tracheotomy is planned), right groin, and *(optional)* dorsal surface of remaining legs (for ECG electrodes); scrub animal using 70% isopropyl alcohol and 100% betadine solution. First, start with betadine and then use alcohol. Alternate between betadine and alcohol for a total of three swabs betadine and two swabs 70% alcohol, ending with 70% isopropyl alcohol. Use circular, inside-to-outside motions when scrubbing incision so as not to contaminate the innermost region. Record vitals: Cover eyes with sterile ophthalmic ointment for lubrication (e.g. Vetropolycin, neo-poly-bac, etc.), insert rectal thermometer (lubricated with K-Y), insert EKG leads subcutaneously (ground to hind leg; negative to left front leg, positive to right front leg) and secure both EKG leads and thermocouples/needles with surgical tape (Durapore™ Surgical Tape, 2.5cm x 9.1m [1 in. x 10 yd.] hypoallergenic; REF 1538-1, NDC 8333-1538-01) monitor sO_2 with pulse-oximeter if applicable. Record values on surgery log and anesthesia record.

2.9.2. ACA Surgical Preparation

Don sterile gloves, drape and open surgical pack in a sterile field to arrange surgical instruments (6 week sterility for a double wrap pack and 3 week sterility for a single wrap pack) (see **Table 2.2** for contents). Included in the surgical pack are: Small vessel scissors (protect tip with yellow pipette), 45^{o} 5/45 sharp forceps (protect with tip of yellow pipette), Blunt; bent 16ga/2.5" needle (may bend, blunt and file needle from IV catheter used for endotracheal catheter) or purchase from Popper & Sons, 17TWx3

custom blunt popper biomedical needle (cat# 7427, http://popperandsons.com/index.asp); Fine, straight rat-toothed forceps (Miltex 6-106; ~1mm or 1/16" width x 5 inches long); 90° curved hemostats (5.5 inch long; Roboz RS 7291), 2 x 8 inch smooth inner surface curved (~90°) forceps (for teasing apart and holding vessels, Paragon #4004-96 http://www.paragonmedical.com/); Curved, blunt scissors for cutting skin and separating skin and muscle (Roboz; RS-6891), 4 inch curved forceps for holding cannula (smooth) (rough forceps will tear up surface of cannula) and 2 ½" forceps (rough), one needle holder, clear plastic drape with hole for throat and loin; (*Optional*) Y-Luer adapter; used to connect both Epi and $NaHCO_3$ to venous cannula line for immediate injection during resuscitation, group of 6 inch cotton tipped applicators (quantity based on surgeon's preference), small scissors for cutting knots, ½" pile of gauze (Flush with saline, clean out blood clots, cover with saline wetted gauze), (*Optional*) Extra, 4 x 23ga blunt connectors, 6x 26ga luer stub adapters (for Tygon tubing), 1 silk needle w/suture (for securing endotracheal catheter to lip after intubation), 4-7 x 15 inch pieces of silk thread necessary for anchoring and tying off vessels during cannulation, 2-3 x 3-0 Prolene sutures for skin closure (3/8 19 mm; FS-2, 18" [45 cm] Non-absorbable), *(Optional)* retractor for throat if doing tracheotomy; IV catheter (BD Insyte™ Autoguard™ Shielded IV Catheter; 14ga x 1.75 inch; 2.1 x 45mm [330 mL/min], REF 381467).

2.9.3. Arterial and Venous Femoral Cannulation

First cannulate the femoral vein followed by arterial cannulation. Flush with heparinized-saline (hep-sal; 0.2mL of 1000IU/mL/30mL vial saline or 6.7 IU/mL) and temporarily secure with sutures (see **Fig. 2.3** & **Fig. 2.4**). Gas sterilized catheters (Tygon®, flexible tubing; Saint-Gobain PPL Corp., 0.375-mm ID, 0.75-mm OD; length 0.029" & wall 0.014"; Norton, Akron, OH; AAQ041889; Lot No. 112046) will be introduced approximately 2 cm into the right femoral artery and vein for acute blood pressure recording, blood sampling, and drug infusion. First begin by making an initial skin incision by lifting skin up away from animal with forceps and carefully cutting skin with a smooth, curved blunt-end scissors approximately 2 cm medially and ≈ 0.5cm distal to the inguinal crease (*hint*: start parallel to inguinal crease in the groin muscle area and directly across from the knee). Use blunt scissors and 'blunt dissection' technique to carefully stretch the skin longitudinally, no more than 1 inch long. Very carefully, separate fascia connective tissue with the blunt end of the scissors, keeping scissors closed and working caudally (in the direction toward the tail of the animal). Be careful as not to damage the portion of the femoral artery that runs on and along the muscle when first making the incision. Using blunt dissection, gently work along the crease of the muscle of the groin area and work underneath that muscle—gently use the forceps in the other hand to lift muscle up and away so that the femoral artery, vein, nerves, and inguinal ligament is revealed (the inguinal ligament is a shiny white band that runs directly above the femoral artery and vein; see **Fig. 2.4b,c**; also revealing the femoral sheath containing the femoral artery, vein, and nerve (Chen et al., 2008).

Next, very carefully separate the femoral vein, artery, and nerve using fine tip, smooth inner surface, ≈ 90° curved tip 8" forceps (Paragon #4004-96 http://www.paragonmedical.com). Add sterile saline directly onto the femoral artery and vein to allow for easier separation and avoidance of desiccation. Avoid damaging the femoral nerves that run parallel and located on either side of the vein and artery—damaging the nerves will result in disabled animals when recovering further hindering their hindlimb movement and gait. It should take less than10 min to fully separate vessels.

Once vessels have been separated, look for any adjacent branching off vessel—it is best to place silk 3-0 thread tie below the branch so as to avoid any excess blood loss when inserting and tying off cannula. First begin with the femoral vein. Take the silk 3-0 thread, fold in half to make a small loop at the bend, and using the fine tip forceps, grasp the loop, slip underneath vein (preferably underneath a branch) and pull through from the other side using forceps in the opposite hand. Stretch out the thread and make a loop around the vessel tied down close to but not actually on the vessel itself. Use curved hemostats to add tension to the thread by placing it on the very ends of the thread and allow it to hang over the animal's knee located closest to the surgeon (*hint*: always start at the proximal end of the vessel when first tying off either the vein or artery). Now that the proximal tie has been completed, do the same exact step for the distal end of the vein. Tie the 3-0 silk thread all the way down to the vessel until 'snug' (not tight). Make between 2-3 throws (knots) and hang over the adjacent knee using another pair of hemostats. Next, make a small, width-wise incision on the vein using small "vessel"

scissors (FST 15000-00, www.finescience.com) or iris scissors or (make sure there is adequate tension on the vein using the silk ties and hemostats; inadequate tension results in blood loss during this step and/or vessel tearing). When making the same incision on the artery, the cut should not exceed 1/3 of the artery circumference in order to avoid tearing (Callaway and Logue 2009). Gently stretch the incision wide enough so that the cannula can fit through the opening 'snugly' and will not accidently slip back out (the vein is more flexible and prone to tearing compared to the artery). Also, periodically check for vessel desiccation and add sterile saline as needed. Carefully and gently insert the heparinized-saline filled cannula (primed) approximately 2cm into the vein (2cm should be indicated by a black mark on the cannula itself prior to sterilization). Cannula advancement should be stopped or adjusted based on blood flow resistance (this is very critical when cannulating the artery due to high blood pressure). Slip cannula as fully as possible into the vessel. It is fine if the cannula is not inserted 2cm into the vessel, but enough so that the cannula will not slip out. While using one hand to hold the cannula in place with the fine tip forceps, use the other free hand to tie down the silk tie onto the vein containing the cannula (*hint*: use the thumb and index finger of the free hand to hold the thread and carefully stretch length-wise to the rat until "snug" fit). As soon as the tie is secured to the vein and cannula tubing, make at least 2-3 additional throws (knots) to secure cannula. Next, tie the proximal end of the cannula to the vein and make 2-3 throws. Repeat the aforementioned steps for arterial cannulation with the exception of starting with the proximal end first. Be very careful of high blood pressure in the artery. If there is a lot of resistance, then carefully flush with hep-saline using one hand while

grasping the cannula with forceps. Once the artery has been cannulated, check for adequate blood flow with small hep-saline flush. Blood should flow back toward the hep-saline syringe (however, do not allow blood to flow back up the syringe or into the pressure transducer).

Mean arterial blood pressure (MABP) will be measured via an indwelling femoral arterial catheter connected to a pre-calibrated Statham pressure transducer and will be recorded continuously. Arterial blood gases and pH, and plasma glucose, will be measured in microsamples (75-100μL) (Total cumulative volume from repeated sampling not to exceed 1.5mL).

2.9.4. Induction of ACA and Positive End-Expiratory Pressure (PEEP)

Immediately paralyze rat after cannulation by injecting 0.3mL vecuronium (1mg/mL) IV and watch to see that animal stops breathing. Immediately connect endotracheal catheter to ventilator (Harvard Apparatus '683' Model Small Animal Ventilator or UGO Basile 7025 Rodent Ventilator). Air is humidified with mucomist (10% acetylcysteine; Roxane laboratories, Cincinnati, OH; NDC 0054-3027-02) with pressure dampener rebreathe bag attached and set approximately to 60 rpm, stroke volume (SV) is adjusted based on body weight (1mL/100g up to 300g max) and typically set between 2.0-3.2mL—anything set over 3.2mL could potentially damage or rupture the lungs. SV $\leq$ 2.0mL/min does not provide adequate expansion of the lung for efficient

gas exchange. Immediately decrease isoflurane to 0.5% and sample blood gas. Adjust blood gas values to within normal physiological values for Po_2, Pco_2, and pH: flow rate of O_2 is typically between 450-500mL/min and N_2O is 1L/min (a 30:70 mixture of O_2/N_2O) while isoflurane is typically maintained between 1.2-1.8% flow rate; Rat Pco_2: 35-40 and Rat Po_2: 100-130. Monitor rectal and head temperature (T_{rec} and $T_{temporalis}$) until between 36.5-37.5°C.

At least 10 min after previous injection of vecuronium, inject vec (0.3mL of 1mg/mL, IV) and FLUSH immediately with 0.3mL hep-sal (6.7 IU/mL), 2 min later disconnect ventilator. OBSERVE THAT ANIMAL IS NOT BREATHING. If animal continues to breathe (>2 breaths) inject another 0.3mL of vec, wait 10min, and repeat this step. If animal is not breathing prepare to resuscitate[2]. (Connect 2 way luer adapter primed with Epi (5ug/mL) and $NaHCO_3$ (8.4%) for immediately delivery; change BP scale to 0-50; turn off isoflurane vaporizer; turn N_2O flow meter to 0 and increase O_2 to 2L/min and respiratory rate to 80 bpm. Record the last vec injection and start of CA or sham procedure and Resuscitation in LabScribe in the marks entry.

2.9.5. Cardiopulmonary Resuscitation (CPR)

Resuscitate by reconnecting rat back to the ventilator, enter "Res" on LabScribe in the marks entry, and immediately inject 1mL/kg fresh Epi (5ug/kg or 10ug/kg; West-ward, Epinephrine Injection, USP). Apply rapid manual chest compressions at a rate of

200/min to the base of the heart (**Fig. 2.4**) until BP is at least 50 mm Hg and is maintained by spontaneously beating heart for at least 10 sec. If not resuscitated after 1 min, give a second Epi dose. Epi administration should help provide ventricular contraction via direct myocardial stimulation and increase heart rate (Westfall et. al., 2006). Once heart rate (HR) is spontaneous and MABP > 50 mm Hg, immediately inject 0.9 cc $NaHCO_3$. Resuscitation is discontinued after 2 min if animal fails to maintain a spontaneously beating heart. Squishing sounds from lung during manual compression is indication of lung injury and may be used as a basis for euthanasia.

Sample blood gases at 10 min of spontaneous circulation (ROSC). After 10 min of ROSC, decrease respiratory rate from 80 to approx. 60 bpm and % O_2 from 100% to 70% in a mixture with N_2O. Continue to sample arterial blood gases (with at least 10 min. between sampling times) until animal maintains stable values for pH, Pco_2, and Po_2 within normal range for this species. Correct acid-base status with sodium bicarbonate and/or the ventilator settings. (Base excess should be >0; 5-10 is normal). If pH<7.3 inject $NaHCO_3$ to adjust pH. If pH is still too low, RR might need to be increased in order to blow off CO_2. Stroke volume must be >2mL, to achieve adequate lung expansion.

2.9.6. Cannulation Removal and Extubation

Once blood gases are normal, remove arterial and venous catheters by tying off artery and vein with 3 throws of 3-0 silk suture. Use Close skin with 3-O prolene, simple interrupted stitch; at least 3 throws. Inject Sterile Saline (1mL/kg, intraperitoneally [IP]). Insert IPTT-300 temperature transponder tag (Bio Medic Data Systems, Inc. [BMDS], Seaford, Delaware; Reorder# IPTT-300) subcutaneously (SC) between the shoulder blades, if not already present. Leave on ventilator until spontaneous breathing begins to fight ventilator. Maintain at 37.0°C (between 36.5 and 37.5°C) for at least 60 min. Animals not able to maintain 36-37.5°C body temperature without heat lamps are placed in a humidified neonatal incubator set at 29.0°C for 12-24hr or until animals regain normal thermoregulation.

Place animal in postoperative cage (with underpad and water bottle provided) and put in neonatal incubator set to 29°C once animal spits out endotracheal catheter on their own (or with some help by the surgeon if catheter is still lodged in throat due to mucus secretions). For the next 8-16 hours directly after resuscitation, DO NOT FEED or PROVIDE ANY FOOD in the recovering animal's cage as this can sometimes lead to accidental death (e.g. their face can be submerged in their food while trying to eat while in a very comatose state and result in an "accidental asphyxiation"). Dehydration will be prevented by giving an IP sterile saline injection (1mL/100g body weight [b.w.]) prior to placing recovering animal inside the incubator (this should be done before temperature

transponder is implanted at the end of surgery) and by also placing a water bottle in their cage.

Copy and file anesthesia records, surgery logs, data sheets, animal care log sheets, and neurological deficit scores (NDS) sheets into IACUC animal quarters and vet services binders located in animal quarters office, B6. Enter blood gas data into Excel, back up LabScribe files to both laptop and designated external hard drive. Monitor a minimum of 3 times daily per post-op care below until animal shows signs of voluntary feeding, cleaning, and is able to easily move around in cage on their own. Record body temp daily and inspect/clean sutures daily up to 7 days and at regular intervals thereafter. Remove sutures at 10-12 days.

2.9.7. Post-Operative Care

Daily feeding, cleaning, and temperature checks will be done on post-operative animals for at least one week after surgery, if not more if doing a longer term study. Wounds are cleaned with diluted (tea colored) betadine and a piece of gauze once per day for 3 days and inspected thereafter. Between 8-16 hours after surgery, signs of distress are to be observed in the rat while they are housed in the neonatal incubator. Rats should also be fed a liquid diet at this time (and a minimum of 3 times per day for the first few days until animal can eat on their own and maintains a healthy weight) using a "spoon-feeding" technique even if they are somewhat able to eat on their own (sometimes seen in some 6 min CA animals). Food will consist of a ~50:50 mixture of ground rodent chow

and sucrose (food grade) and dissolved in tap water to make a dilute solution, resulting in a liquid soup mixture that is placed in a small petri dish for their consumption—usually use the bottom lid of the petri dish in order to avoid any necessary spills during feeding (see **Fig. 2.6**). If unable to self-feed, animals will be then be fed by gavage (1mL/100g b.w.) a minimum of 3 times per day (stomach volume ≈ 3 ml in a 300g rat and gastric emptying time is about 45-60 min). These animals will be fed the diluted chow soup mixture drawn into a 3 cc syringe using a curved gavage needle (≈ 11 cm long, 16ga x 1 ½", blunt-ended, with bulb diameter of 4.0 mm (Waynforth and Flecknell 1992)). Caution should be given to avoid clogging the gavage needle by suctioning up the liquid portion of the chow soup and avoiding suctioning up food particulates. "Spoon-feeding" will be done for animals showing any "swallowing" response. Place the tip of the gavage needle in their mouth barely past their teeth, and gently push the liquid food contents down in their mouth while waiting for their "swallowing" response. Rats should only consume as much as food as they will swallow (usually a strong appetite response immediately after the 8-16hr post-operative period, but dependent on individual variation). Animals not consuming at least 3ml/100g are fed by gavage. Beginning at 8-16hr post-op body temperature is recorded and animals are monitored for neurological impairment and scored per "Neurological Deficit Scores (NDS)" daily for the first 7 days and at regular intervals thereafter (e.g. for 22-day studies, NDS done every 3 days after day 7: Day 10, Day 13, Day 16, Day 19, and Day 21).

Although the severity of brain damage is not expected to produce death, inter-animal variation makes death a possibility. Animals will be monitored, fed by gavage

(1mL/100g b.w.) and given supportive care as needed at least 3 times per day. Severity of brain damage may be assessed from neurological examination. Animals showing severe neurological impairment including coma should not be euthanized because eliminating most severely affected individuals will bias final assessment of histology. The number of animals that die will be noted, however, these animals are excluded from histological examination of tissue because death does not allow for in situ fixation of the tissue. Vet services should be contacted in case of complications encountered during regular work hours or during after-hour, weekend or holiday care (e.g. wound infection or unusual behavior by animal).

2.9.8. Neurological Deficit Assessment: Behavioral Scoring, Perfusion, & Histology

A neurological deficit score (NDS) will be performed daily for 7 days (or up to 22 days if a long-term study) after global cerebral ischemia (see **Appendix E** for NDS worksheet). The total NDS consists of five components: consciousness and respiration, cranial nerve function, motor function, sensory function and coordination (leg/tail movement, cleaning, depth perception, righting reflex) and for motor and sensory function as previously described (Katz et al., 1995). The NDS range is a scale between 0 (normal function) to 100 (brain dead). To have consistency in results between animals, NDS scores should be done by one person. NDS scores may also be taken 1 or 2hr after resuscitation to assess acute impairment.

Neurohistopathology requires a minimum of 3 days to develop and is more apparent 7 days after ROSC. Neuroscores are collected up until animals are euthanized and brains collected for histopathology. Brains are perfused in situ via the heart using filtered saline (0.9% saline; 9g NaCl/1000mL diH2O) for 1 min. Saline is followed immediately by 19 min. of FAM (100mL of 36% formaldehyde, 100mL of 99.5% acetic acid-glacial, & 800mL 99.8% methanol per 1 Liter of FAM solution). Briefly, animals are anesthetized with isoflurane (5% mixed with 100% medical [Rx] grade O_2 gas, delivered at 1.5L/min; and maintained at a surgical plane at approximately 3% isoflurane). The Thoracic cavity is opened, the descending aorta clamped, the right atrium is punctured, and solution is then delivered into the left ventricle using a peristaltic pump (MasterFlex® Economy Drive "Easy-Load®" Pump, Model# 77200-62; MasterFlex® Precision Tubing, Lot # 109548, Reorder # 06429-15) at a rate of ≈ 80mL/min for rats weighing between 250-350g.

After fixation is complete (20 min. total), animals are decapitated using a rodent sized guillotine and heads are placed in a jar containing FAM solution for 24hr at 4°C. After 24hr, heads are rinsed with distilled H_2O for 10-15 min (to relieve FAM odor and prepare for next solution), brains are carefully removed and placed into a 45 mL centrifuge tube containing approximately 25 mL of FAM for another 24hr at 4°C. Brains are placed in 70% ethanol solution and stored indefinitely at 4°C until ready to be trimmed and sectioned into coronal brain blocks for paraffin embedding.

Using a brain matrix apparatus (Braintree Scientific Inc., Braintree, MA; B5-A 6000C), the brain is trimmed into two 3 mm section blocks for the hippocampus and striatum regions (-3.80 mm from bregma; -1.80 to -0.80 mm from Bregma, respectively; **Fig. 2.8**). Coronal sections of 8μm were stained with hematoxylin and eosin (see **Appendix H, I**). Rat brain sections examined contained the hippocampus at approximately -3.8 mm posterior to bregma. Immediately placed into a cassette (for hippocampus placed caudal-side down and for striatum placed rostral-side down inside cassette), then into a jar filled with 70% ethanol until ready for embedding. The embedding process is as follows: (30 min for each) 70% isopropyl alcohol x 2, 80% isopropyl alcohol x 1, 95% isopropyl alcohol x 2, 100% isopropyl alcohol x 3, Toluene x 2 paraffin 56°C x 2, and 20 min in vacuum embedder. Slides are stained in Hematoxylin for 5 min. and in eosin for 1 min. (see **Appendix I**). The whole cycle should take approximately 22 hours to complete.

2.10. ACA Troubleshooting

Table 2.3 provides a detailed list of various problems that can arise during the ACA surgical procedure and post-operative care. The most common problems that occur during ACA include the following: ineffective anesthesia, inadequate oxygenation after ROSC, difficulty inserting endotracheal catheter into trachea, swollen epiglottis/vocal cords during intubation, arterial/venous clotting during femoral cannulation, poor cannulation thread tension, inability to resuscitate animal, mucous in lungs after

resuscitation, too low/high temporalis/rectal temperatures, no ECG/MABP signal in LabScribe Acquisition software, excessive porphyrin secretion, wound infection of the cannulation site during post-care, etc.

2.11. Conclusion

In conclusion, the ACA rat model provides reproducible brain injury in a controlled laboratory setting and mimics neuronal injury seen clinically (especially in perinatal/pediatric populations). The ACA model is easier to implement than the 4VO and 2VO with hypotension animal models and provides a wider therapeutic window to investigate neuronal pathology. Although the ACA model is a great tool for studying the excitotoxic mechanisms of global cerebral ischemia, there are some disadvantages as well. The ACA model is a very laborious and time-consuming process from surgical implementation and technique to intensive post-operative animal care 1 week after CA injury.

2.12. Acknowledgments

This study was supported by National Institutes of Health grant NS41069-06, US Army Medical Research and Materiel Command (# 05178001), UAF Center for Research Services, and Flint-Hills Resources. I would like to personally thank Dr. June Sun, Heather Crispell, Dr. Tulasi Ram Jinka, Jasmine Davis, and Lindy Smith for surgical

assistance, post-operative care monitoring, and moral support; Jeanette Moore, Zachary Carlson, and Joel Vonnahme for blinding experimental drug treatments; Dr. Kelly L. Drew, Dr. June Sun, and Kimberly E. Iceman for initial LabScribe and iWorx equipment setup, data analysis, and troubleshooting; and finally Lori K. Bogren for additional technical assistance. I would also like to thank my colleagues at the Department of Neurology (D4-5), University of Miami Miller School of Medicine, Dr. Kunjan R. Dave, Dr. Miguel A. Perez-Pinzon, Isabel Saul, Ami P. Raval, Guillermo Fernandez, for further technical assistance and troubleshooting.

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Table 2.1. Models of Global Cerebral Ischemia

4VO Advantages	4VO Disadvantages
▪ Reversible forebrain cerebral ischemia (rats)[2]. ▪ Metabolic & morphological studies ▪ Can implement on awake, freely moving, or anesthetized rats[4,5]. ▪ 50-75% success rate[2]. ▪ Useful to test drugs for neuroprotective activity. ▪ Cell death closely mimics human ischemia[2]. ▪ Opportunity to measure physiological without use of anesthesia or hypotension[1,5].	▪ Rat strain choice is critical (wistar preferred; not all strains adaptable) [1,4,5]. ▪ Harder to implement (2-day procedure) ▪ Histopathology variability from procedure, especially between animal strains and ischemia duration[2,5]. ▪ Difficult to determine vertebral artery location and occlusion of these vessels are critical to outcome success[2]. ▪ Unsuccessful outcome occurs in 25% rats due to respiratory failure[4]. ▪ Inconsistency between outcome yields; 50% survival to first-stage procedure even in best-case scenario[4].
2VO w/hypotension Advantages	**2VO w/hypotension Disadvantages**
▪ Model of forebrain ischemia[2,4]. ▪ Suitable for chronic survival studies[2]. ▪ Produces selective and delayed neuronal death and reliable injury for behavioral and histological assesstment[5]. ▪ Simpler surgical preparation than 4VO[2]. ▪ Fast onset of ischemia and reperfusion[2]. ▪ Sufficient forebrain ischemia from hypotension (50 mmHg via bleeding or hemorrhage)[4].	▪ Physiological variability is expected from the procedure and beyond surgeon's control[5]. ▪ Anesthesia and hypotension drug-interaction can confound data results[2]. ▪ Minor fluctuations around 50mm Hg from reduced blood flow can produce variable neuropathological results[4]. ▪ Outcome success dependent upon adequate hypotension production[4]. ▪ Behavioral changes subsequent of occlusion cannot be assessed since this model cannot be used in awake animals[4].

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Table 2.1 Continued…

ACA Advantages	ACA Disadvantages
▪ Produces complete, 'true' global ischemia in both brain and peripheral organs[3]. ▪ Easy to implement & control physiologically. ▪ Not necessary to monitor cerebral blood flow. ▪ Histological damage seen after several days from injury (first appears 1-2 days after resuscitation up to 6 weeks later)[3]. ▪ Relatively cost-effective in terms small animal (rodent) use, supply, and duration of surgery[2,3]. ▪ Minimally invasive. ▪ Only about <1% of rats will develop ventricular fibrillation during cardiac arrest increasing CPR survival[3]. ▪ Validated model in terms of severe neurological deficit production similar to human disease resolved partially or completely over a couple of days[3]. ▪ Reproducible & reliable injury[3]. ▪ Acute time investigation of injury. ▪ Able to use sophisticated neurosensory and motor behavior assessment as a measure of ischemic injury outcome[2]. ▪ Mimics clinical scenarios in human diseases (e.g. 'birth asphyxia', transient coma, sensorimotor deficits, hypoxemia, acidosis, choking, systemic inflammation, hyperglycemia, hypercotisolemia, near-drowning, and near-SIDS)[3].	▪ Labor intensive (e.g. post-operative care); not for high-throughput studies[3]. ▪ General animal welfare (paralytics and no analgesics) concerns. ▪ Results vary depending on experience of surgeon[3]. ▪ Not clinically similar to ventricular fibrillation, the most common cause of CA in adults. ▪ Post-operative rats may be in a coma for several days after cardiac arrest[3]. ▪ Progressive weight loss during 1st week of post-operative care is expected despite careful monitoring and feeding[3]. ▪ Swelling can occur from multiple intubation attempts (>2) leading to respiratory problems or even mortality (especially after extubation)[3]. ▪ Deaths generally occur between1-5 days after resuscitation (e.g. secretion build-up)[3]. ▪ Individual variation occurs between surgeons and surgical preparation involves multiple-steps[3].

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Table 2.2. Setup and types of packs used during asphyxial cardiac arrest surgery.

Early Setup
☐ Color-coded syringes for injectables: 5 x 1cc sterile syringes (2 filled with heparinized-saline, 1 for Vec, 1 for $NaHCO_3$, and 1 for Epinephrine); 1 x 10 cc sterile syringe filled with sterile saline (see Appendix D); e.g. of color-coding: red = Epi, orange = hep-sal, green = saline, yellow = vec, & white = sodium bicarbonate
☐ 1-2 Surgical Tape (Durapore™, 1"x 10yd [2.5cmx9.1m] REF 1538-1)
☐ Rodent Intubation, tilting workstand (www.hallowell.com; No. 000A3467)
Intubation Pack (autoclaved)
☐ 2 x 15" cutting sutures (for securing catheter to lip after intubation)
☐ 1 x 4" straight-edge forceps
☐ Rat speculum (plastic), autocalveable (www.hallowell.com; No. 210A3496) attaches to head of otoscope)
☐ Mouse lidocaine applicator (www.hallowell.com; No. 210A3496)
☐ Needle Holder
☐ Custom made Blunt-end, bent 16ga/2.5" needle (may bend, blunt and file needle from IV catheter used for endotracheal catheter) or purchase from Popper & Sons, 17TWx3 custom blunt popper biomedical needle (cat# 7427, http://popperandsons.com/index.asp)
Cannulation Pack (autoclaved and goes inside CA surgical pack)
☐ 2-3 x 3-0 Prolene sutures (Ethicon®, 3/8, 19mm diameter, 18" [45cm] polypropylene cutting suture; FS-2, 8665; nonabsorbable) (optional: if expired, autoclave and place in pack; if sterile can open during surgery)
☐ 4-6 x 3-0 (or 6-0) black silk suture thread (USP 3/0 Silk Suture; Havel's Inc; 100 yd waxed; 47-ASB-54; LOT: 6253); used for tying cannula to vessel
Cardiac Arrest Surgical Tool Pack (autoclaved)
☐ Adson-Brown Forcepts (www.finescienc.com, FST 11627-12) for holding skin during initial cannulation skin incision
☐ Small "vessel" scissors (protect delicate tip with tip of a small pipette; www.finescience.com, FST 15000-00)
☐ "Dumont" 45°, #5/45 sharp, dumoxel std. tip forceps (protect with tip of yellow pipette); (www.finescience.com; FST 11251-25); used to grasp delicate vessel while inserting cannula)
☐ Fine, straight rat-toothed forceps (Miltex 6-106; ~1mm or 1/16" width x 5 inches long)
☐ 90° curved hemostats (5.5 inch long; Roboz RS 7291)
☐ 2 x 8 inch smooth inner surface curved (~90°) forceps (for teasing apart and holding vessels); (Paragon #4004-96 http://www.paragonmedical.com/)
☐ Curved, blunt-end scissors for cutting skin and separating skin and muscle (Roboz; RS-6891)
☐ 4 inch rat tooth forceps for holding cannula and 2 ½" forceps (rough)
☐ 12"x12" clear, sterile plastic mouse drape (Sterile Econ-O-Pak™ , Gepco® Reorder# 1212CPSTF); (not autoclaved with pack & added to pack prior when opening into sterile field)
☐ (*Optional*) Y-Luer adapter (for quick delivery of Epi/$NaHCO_3$ during resuscitation; www.qosina.com, Qosina 83016)
☐ Pile of cotton tipped applicators (Puritan®, 16" [15cm], REF 806-PC; www.puritanmedproducts.com)
☐ Small, "Iris" scissors (delicate, straight edge, 10.5cm; www.finescience.com; FST 14060-10) for cutting knots
☐ Pile of gauze sponges (8 Ply, Kendall Curity™, 2"x2"[5cmx5cm), REF 2146, non-sterile, USP Type II Gauze)
☐ 2 x microscope handle covers (sterile surgical masks w/earloops work best; "DuPont™", Sierra® General Mask, Part No. 733-OBU-00-0300BB, Lot No. M00075808)

Table 2.3. Troubleshooting guide for asphyxial cardiac arrest model.
ANESTHESIA
Difficulty maintaining or inducing a "deep" enough anesthesia in rats. • If the rat is moving around or fits against the ventilator, then slightly increase anesthesia delivery. Isoflurane should be set at the lowest possible delivery setting that causes the rat to be sedated (anywhere between 1.0-2.5% isoflurane delivery on vaporizer unit works best to maintain anesthesia for 230-300g rats). Isoflurane can even be increased to 2.5-3.0% to allow for "deeper anesthesia", but should not be set for longer than 2 minutes at a time because an overdose can accidently occur (it is imperative to watch for breathing patterns and consistency).
ECG
Noisy Signals or no ECG displayed on LabScribe Data Acquisition Software. • Make sure ECG leads are placed subcutaneously in hind and forelimbs (and not in the muscle). Reinsert ECG leads in different spots until a signal is displayed (won't get signal even if only one lead is wrongly positioned). Turn off heat lamps prior to initial insertion of leads (lamps increase noise on ECG and interfere with LabScribe Data Acquisition software; there should still be a signal, albeit a 'noisy' signal). Make sure the settings for "ECG transducer box" are set to 0.1 or 1. Leads should be placed with little to no movement (be still as possible to avoid signal interference).
CANNULATION
Difficulty advancing cannula inside arterial vessel. • Carefully loosen tension of knots (it might be too tight): place smooth curved hemostats (45° angle) underneath knot just above cannula tubing (add saline to help loosen knot). This lightly loosens knot without risk of cannula slipping out from high BP, but enhances natural flow. Correct tension is CRUCIAL for obtaining a successful cannulation.
Difficulty inserting cannula. • Check tension of cannula thread used to crease and occlude. Readjust tension if necessary, to fully occlude flow through cannula. Also check the size of vessel incision (hole)—should be large enough for cannula to fit through. Tip: bevel cannula end (cut at a slant angle) and insert cannula through hole with bevel tip facing toward opening. This should make it easier to insert cannula through vessel.
Unable to obtain any/enough blood from artery for blood gas sample analysis. • Possible clot in artery—flush with Heparinized-saline; check cannula tubing/pressure transducer for air bubbles and/or leaks.

Table 2.3 Continued…
RESUSCITATION (ROSC)
Rat unresponsive to CPR (manual heart massage) as indicated on LabScribe data acquisition software. • Move temperature-controlled heat lamps ≈ 2-3 min. prior to resuscitation (prevents overheating of the heart & more effective CPR; hypothermia is very protective against CA). Ensure proper index finger & thumb placement directly on heart (not lungs). Readjust hand/finger placement until a 'strong' response (MABP spikes) occurs during CPR. Minimize the distance between thumb and index finger on heart. Any lung damage will decrease outcome of survival.
Very low pH from blood gas sample. • Inject an additional $NaHCO_3$ (sodium bicarbonate) based off of 1mL/kg (e.g. if rat weighs 280g, give 0.28 cc or mL of $NaHCO_3$) depending on blood gas pH value.
TEMPERATURE
Error message or "nPt" on Omega® temp controllers won't turn on. • Temperature probes for that specific temp controller unit has "died" and needs to be replaced and recalibrated (Calibration: temperature and time controlled tests to ensure 0.1-0.2°C std. error b/w temporalis and rectal probes).
Low head and/or rectal temperature readings. • Temporalis probe for head temp should be placed correctly inside the temporalis muscle (not subcutaneously). Rectal probe should be placed approx. 3-4 cm inside the anus. Adjust heat lamps and place closer to rat (no >6 in. so as to not burn skin).
Too high (hot). • Move lamps (and other heat sources) further away from body. Best to avoid overheating by constantly monitoring temp. Tip: it is much easier and faster to warm a rat than it is to cool the rat down.

Table 2.3 Continued…
INTUBATION
Difficulty intubating animal. • DO NOT ATTEMPT TO INTUBATE MORE THAN TWICE (otherwise epiglottis will become swollen and be more likely to close up tighter due to irritation/inflammation). Make sure rat is deeply anesthetized (e.g. most rats weighing 240-300g generally require 2 min. of Isoflurane set at 2-2.5%). If a strong gag (uvula) reflex occurs, then rat is not deeply anesthetized for proper intubation.
Swollenness and/or bleeding around vocal cords. • Properly lubricate laryngeal folds surrounding epiglottis (and epiglottis too) prior to first intubation attempt so as to not easily irritate area—vocal cords are very sensitive and prone to agitation with repeated endotracheal catheter insertion. Use cotton swabs to gently stop bleeding. Reapply lubricate gel if necessary and avoid over-lubrication, which can block the animal's airway passage and cause hypoxia onset.
"Squishy" or "gurgling" sounds occur in rat during intubation. • "Squishy" or "gurgling" sounds occurring in rats while intubating generally means that their vocal chords and/or epiglottis has become irritated and/or inflamed (e.g. probably agitated from attempting to insert the endotracheal catheter). Make sure to put enough lidocaine gel on the lidocaine applicator BEFORE applying to the laryngeal folds (the little whitish bands directly above the vocal chords). This should "numb" the area enough so as to not cause a "gagging reflex" in rat during intubation thus causing further trauma to the larynx area. If the rat is moving or has a "gagging reflex" while you intubate, then slightly increase anesthesia delivery (2.0-2.5% isoflurane works best to maintain anesthesia in 230-300g rats; 2.5-3.0% isoflurane works better for "deeper anesthesia" but do not set for longer than 2 minutes at a time because an overdose can occur). The rat should have consistently slow, even, and deep breaths to ensure a good intubation response.
Two unsuccessful intubation attempts occur. • In order to increase the likelihood of recovery from asphyxial cardiac arrest and resuscitation, it is best to only attempt two endotracheal intubation attempts--more than that increases the chances that the rat may not survive cardiac arrest and resuscitation. Another option is to have two rats available for any given surgery–meaning that if you fail intubation with one rat after two tries, then you will have another rat available to use. After two failed intubation attempts, the rat can be returned to its home cage (after it recovers from anesthesia, usually ≈10-15 min.) and allowed to recover in about 1-2 weeks so that it can be used for CA surgery again (the throat area should have healed within that period of time).

Table 2.3 Continued…
POST-OPERATIVE CARE
There is light to dark reddish urine and/or tear secretions from eyes, nose, mouth, and anus that appear to be similar looking to blood. • These are called *porphyrins* which have a "blood-like "appearance, but are not blood. Porphyrin production is a direct indicator of physiological stress experienced by an animal. This generally occurs within the first 3 days or so of recovery from cardiac arrest. Be attentive to secretions and rat behavior during this time and give extra supportive care (e.g. use a wet gauze to gently remove secretions from eyes, nose, mouth, and penis) as needed.
Rat's behavior is very agitated and irritable; very difficult to handle while "spoon-feeding" using gavage needle (e.g. squirm and fight against feeding). • If rat is very ambulatory and fights during handling and/or feeding, this is a good indicator that the rat appears to be "feeling better" and doesn't need as much supportive care. Rat should be placed on a dry chow diet or given chow soup (50:50 chow: sugar mix in tap water) in a petri dish to administer self-feeding. If rat is unable to eat much on his own or fights a little bit, be sure to hold animal in a gentle but firm grip (e.g. place hands around and underneath rat's forelimbs [armpits] for a 'stronger' grip) and place the bulb of the gavage needle into the mouth and let the rat "suck up" the liquid chow on their own. GENTLY and SLOWLY release contents into the mouth barely past the rat's teeth and NOT DOWN THE THROAT (be sure to watch for animal's own swallowing reflex for clues when to increase or decrease amount of feeding).
Infection of suture area on cannulated leg. • ALWAYS check wound area and clean with dilute betadine solution ONLY as often as possible, but at least once per day. If infection occurs, be sure to clean the area even more so with betadine. If the infection appears to be "glossy" and/or a darker color, then immediately consult with Vet Services as how to further proceed (Baytril, IP, may need to be given depending on severity of infection and veterinary oversight).

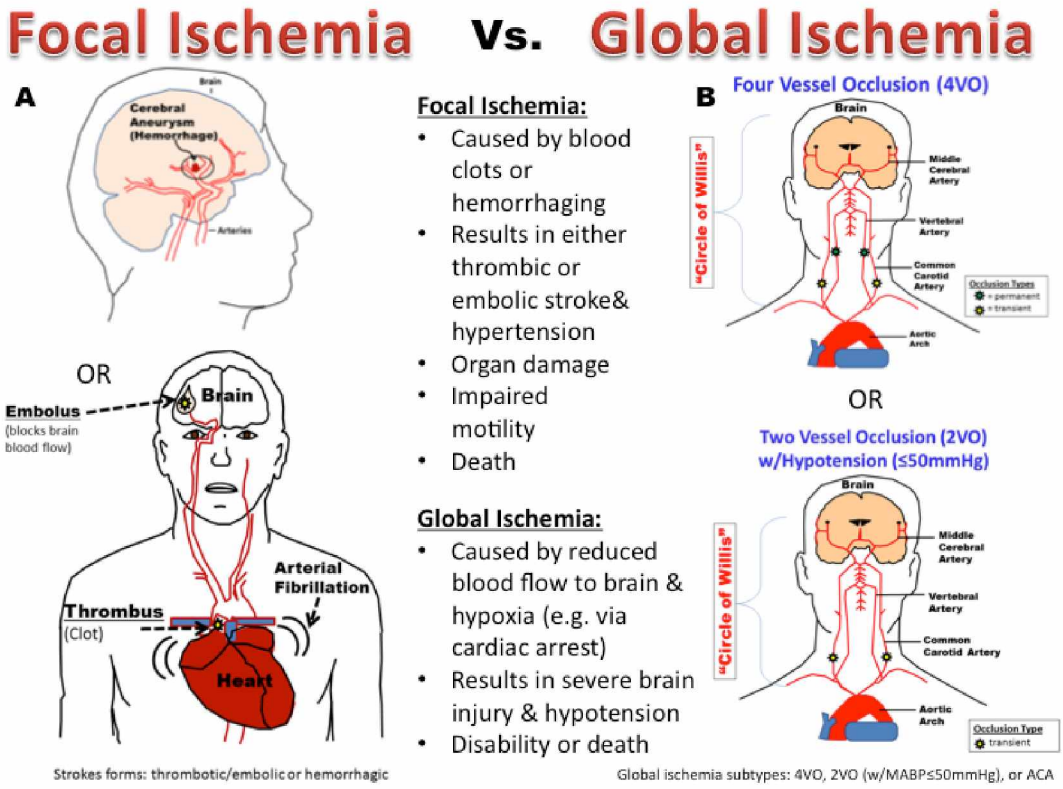

Figure 2.1. A comparison shown between *focal* (stroke) and *global* (asphyxial cardiac arrest) cerebral ischemia models. **(A)** Focal models: **i.)** Thrombotic or embolic stroke, or **ii.)** Hemorrhagic stroke. **(B)** Global ischemia models: **i.)** four vessel occlusion (4VO), **ii.)** two vessel occlusion with hypotension (2VO), and **iii.)** Asphyxial cardiac arrest (ACA).

Figure 2.2. Cardiac arrest facility and equipment setup **(a-f)** shown. Instrumentation **(a)** includes a mechanical ventilator **(b)**, a surgical microscope **(c)**, tray for surgical instruments **(d)**, an isoflurane vaporizer unit (e) and iSTAT analyzer **(f)**.

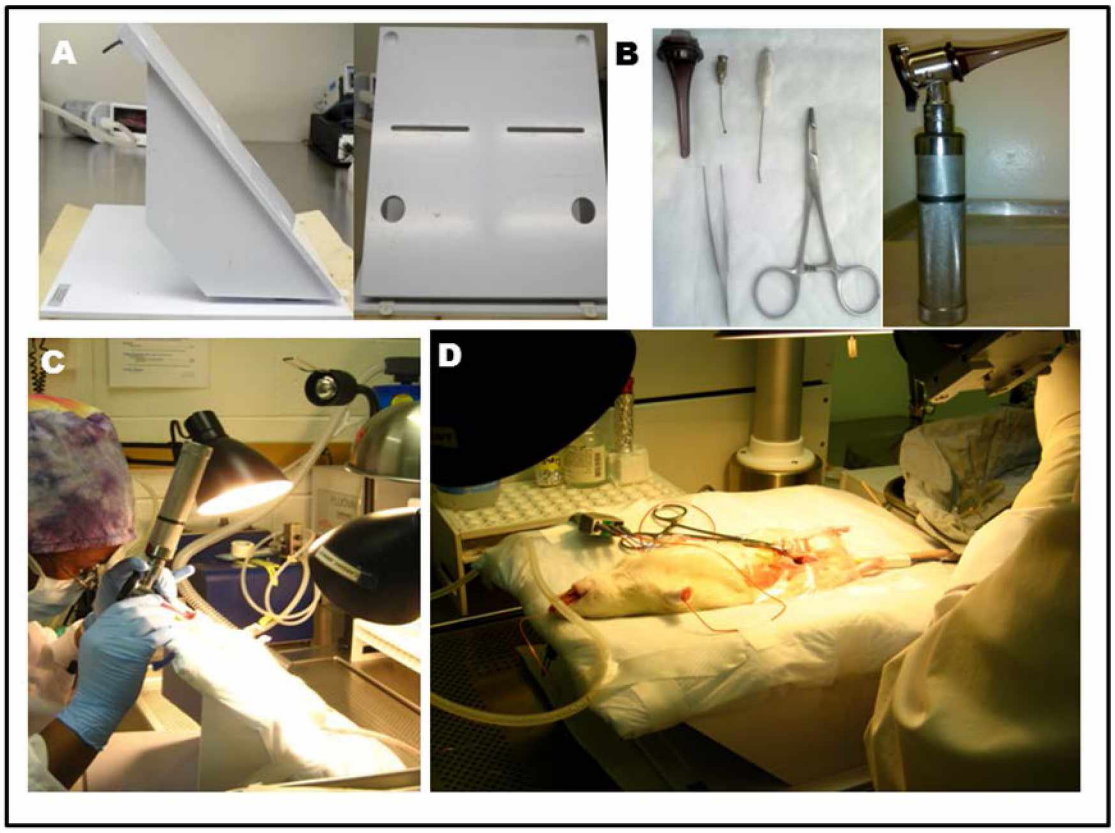

Figure 2.3. Rat intubation stand (**a**), endotracheal intubation tools and otoscope (**b**), and animal preparation for recording physiological vitals (ECG, temperature, and intubation) prior to induction of asphyxial cardiac arrest (**c-d**).

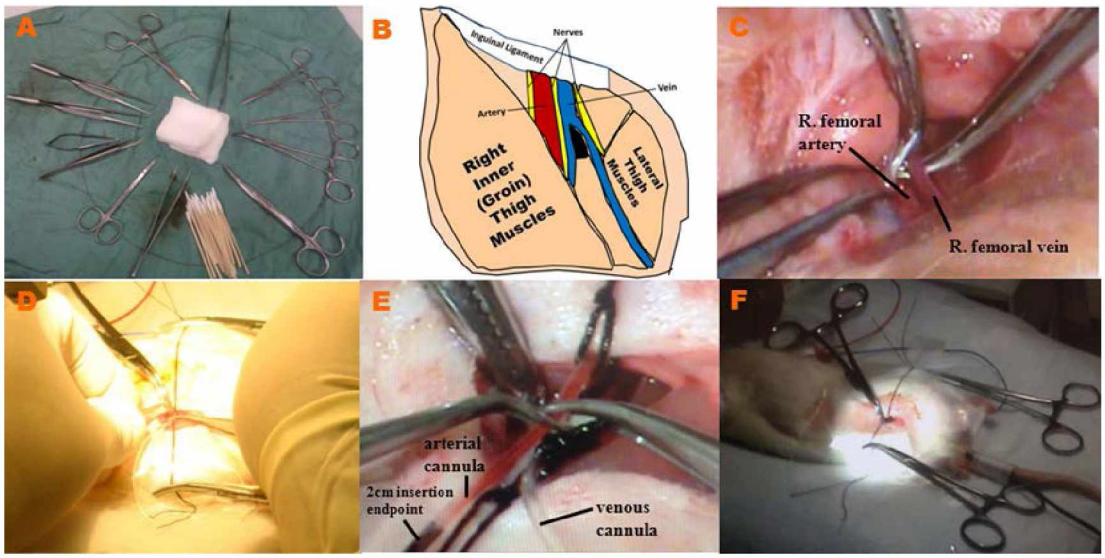

Figure 2.4. Cannulation equipment, diagram, and procedure shown. Sterile tips are oriented towards the center of the sterile field **(a)**. Orientation of femoral artery and nerves **(b)**. Gentle removal of fascia connective tissue reveals the location of the right femoral artery and vein **(c)**. Insertion of arterial cannula into the r. femoral arterial **(d)** using fine tip curved forceps **(e)**. Completed femoral artery and vein cannulation **(f)** with tension applied using 3-0 silk suture thread to each vessel.

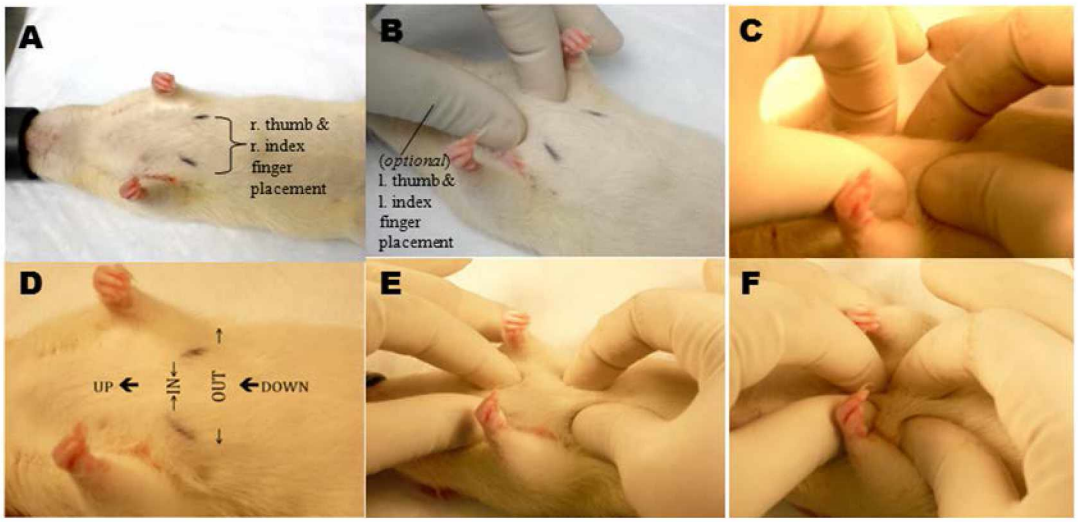

Figure 2.5. Finger position and hand movement shown for the implementation of manual chest compressions during resuscitation of a rat. (**A**) Black marks indicate index finger and thumb position on rat heart for proper manual chest compressions. (**B**) (Optional) The left index finger and left thumb can also be used to support the direction of chest compressions made by the right hand. (**C**) Initial left hand and right hand index finger and thumb placement during CPR. The distance between the right thumb and right index finger on heart should be minimized in order to ensure the best CPR outcome. (**D**) Directional movement shown for effective chest compressions. The right index finger and right thumb should move in an "inward-outward" position followed by an "upward" motion starting at the base of the heart where the ventricle is to effectively pump blood back into the heart from the ventricles up through the atria. (**E-F**) Finger chest compression movement is shown. Right thumb and right index finger should move in an "upward-outward" sliding motion (**E**) starting at the apex of the heart (bottom left ventricle) and sliding towards the base of the heart (up to the atria) (**F**) to pump blood back into the atria of the heart.

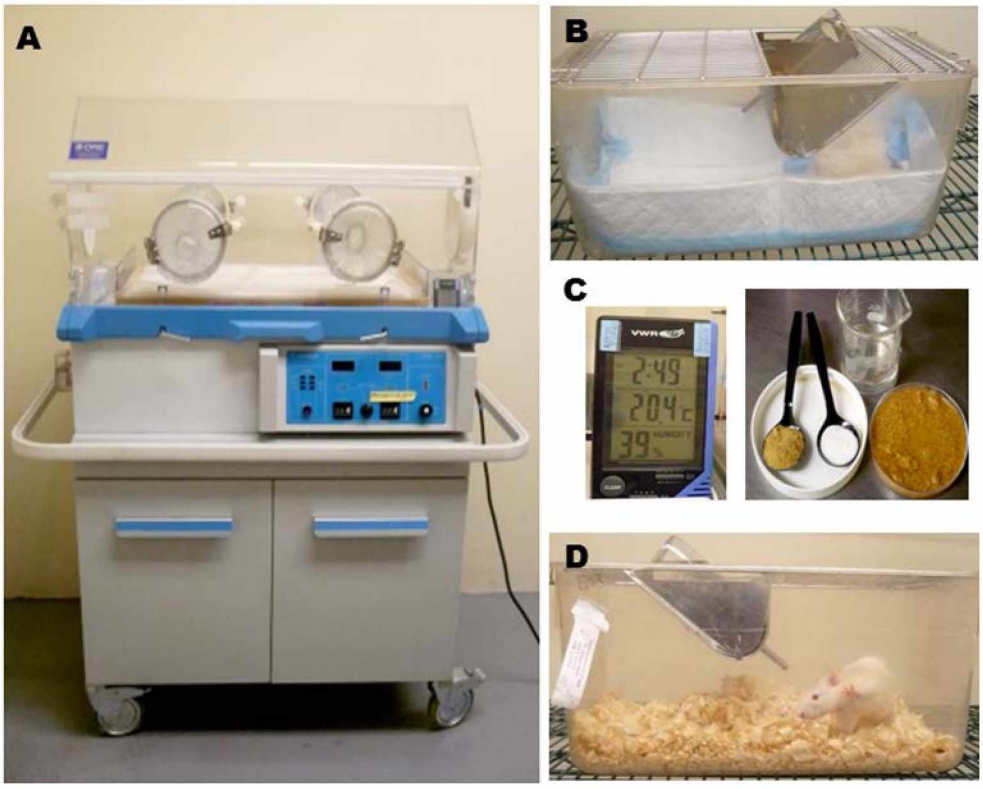

Figure 2.6. Post-operative care food and housing for rats recovering from post-cardiac arrest (CA) surgery. **(A)** Neonatal incubator used to house rats 24hr after resuscitation. **(B)** Post-op "recovery" cage consisting of a water bottle, an underpad, and cotton. **(C)** (Left) temperature and humidity monitor for neonatal incubator; (right) Rats unable to feed on their own and/or are unable to adequately consume enough food are fed a soupy mixture consisting of 50:50 dry rodent chow (finely grounded) and table sugar mixed with water. Chow soup is then placed in a petri dish for consumption. **(D)** Regular housing consisting of wood shavings for rats prior to cardiac arrest surgery and/or after recovery from CA once animal shows signs of normal self-care (e.g. self-feeding, grooming, hiding under padding, etc.).

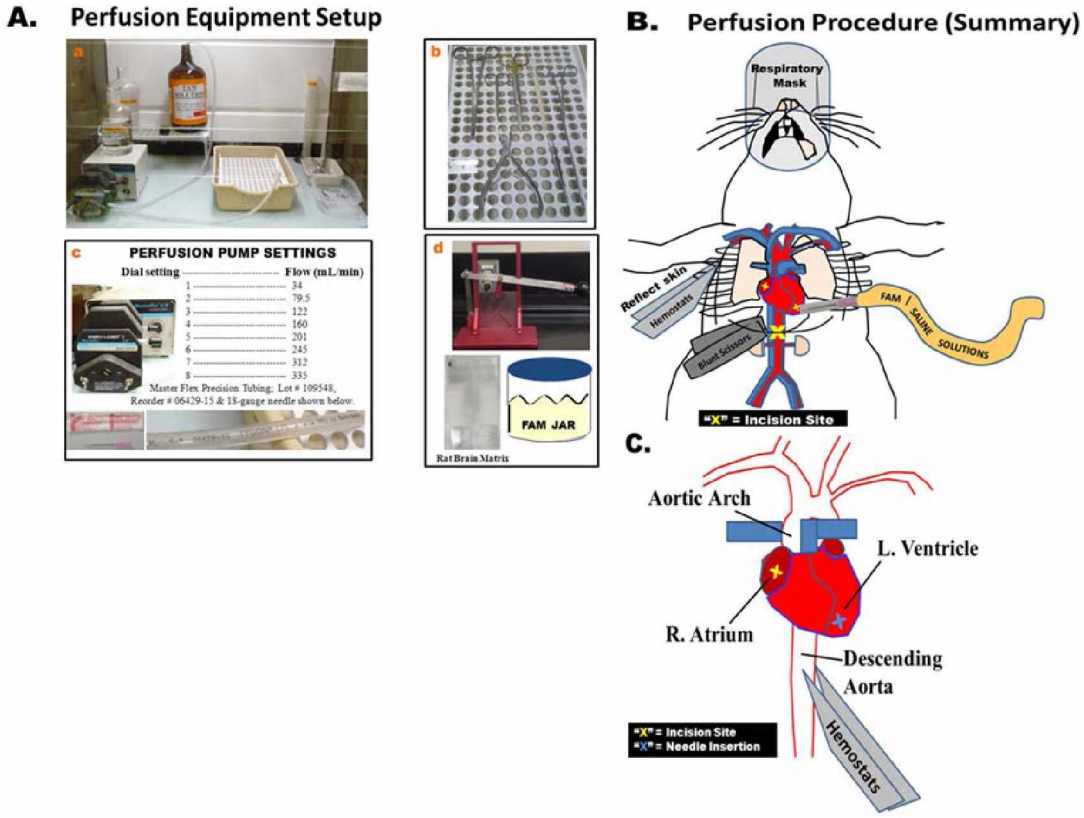

Figure 2.7. Perfusion equipment and procedure used to collect rat brains necessary for neurohistopathology assessment. **(A)** Perfusion Equipment: Biosafety hood with air filters, perfusion pump system (Master Flex Precision Tubing; Lot # 109548), and perfusion tray (9x13inch plastic pan with slits) which allows for drainage of blood and solutions (filtered saline solution [9g NaCl/1L diH_2O]; ≈ 3-4L of FAM solution [Per 1-liter: 100mL 36% Formaldehyde, 100mL 99.5% acetic acid glacial, 800mL 99.8% methanol]), and surgical tools (blunt-tip surgical scissors, 2 x hemostats, sharp-tip small iris scissors, rougeurs. **(B)** Perfusion Procedure: **i.)** Anesthetize with 5% isoflurane (≤2min), **ii.)** Maintain rat on 2-3% isoflurane to ensure deep anesthesia (4-8min), **iii.)** Cut directly below xiphoid process & cut in a "V-shape" formation along the ribcage to expose the heart and lungs, **iv.)** Use a hemostat to clamp off descending aorta, **v.)** Insert 18ga needle into left ventricle, **vi.)** Use iris scissors to cut a small hole in right atrium, **vii.)** Set perfusion pump to "2" & fix brain with saline for 2min → immediately fix with FAM (19min), **viii.)** Quickly decapitate rat with guillotine & use rougeurs to remove surrounding muscle near brain, **ix.)** Store head in FAM jar (24hr), **x.)** Carefully remove brain & store in FAM (24hr), **xi.)** Rinse brain w/diH2O & store in 70% ethanol until ready for trim & paraffin embedding.

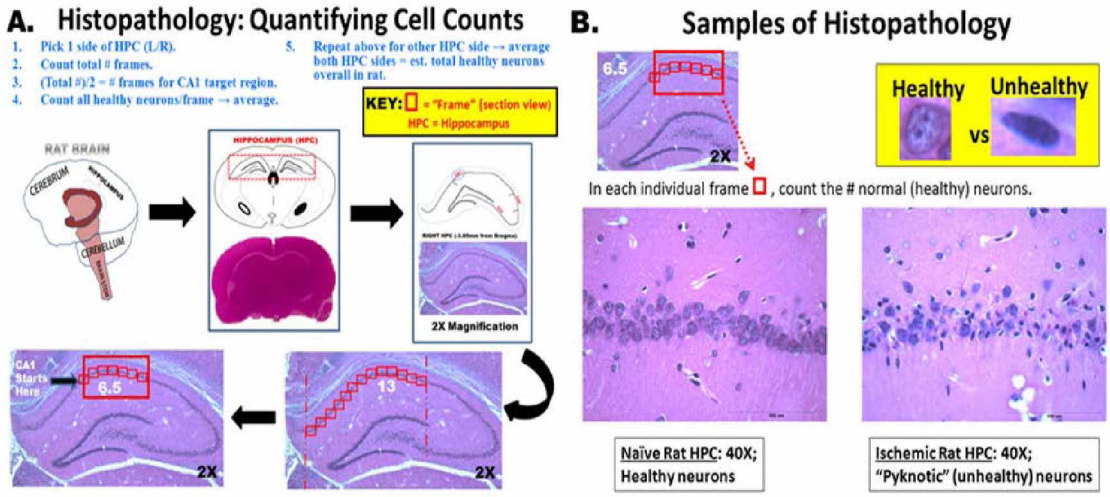

Figure 2.8. Neurohistopathology hippocampus coronal brains sections of rat for neurological deficit severity assessment. **A.)** Schematic shown for quantifying cell counts in a rat brain (hippocampus, HPC). **B.)** Sample of "healthy" neurons in a naïve rat versus "ischemic" (*pyknotic*) neurons in a cardiac arrest rat in the CA1 region of the hippocampus in rat brain.

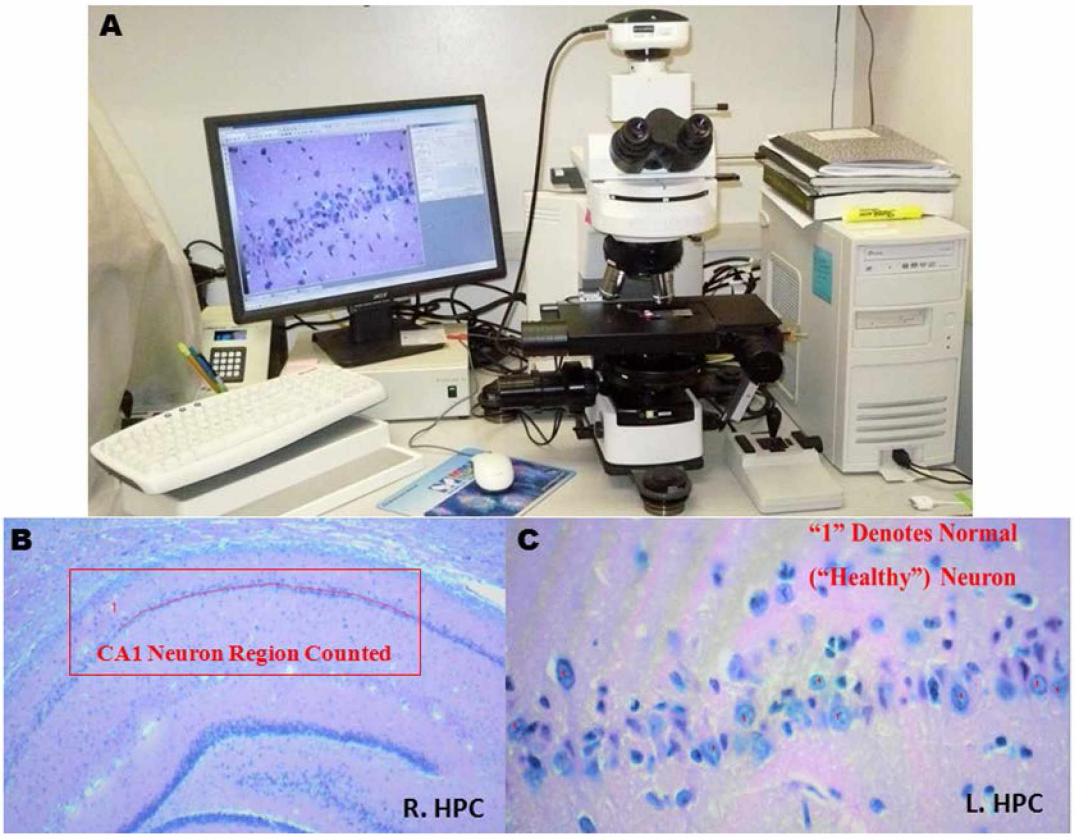

Figure 2.9. Microscope equipment assessment of histopathology (neuronal deficit expressed as the number of "healthy" [normal] hippocampal neurons per mm of CA1). **A.)** Computer, Nikon Eclipse 80i Upright microscope, and MetaMorph Software version 7.1.2.0 used for neuronal cell count analysis of rat neurons. **B.)** Close-up view of CA1 hippocampal "healthy (normal)" neurons in rats. **C.)** CA1 HPC neuron counts shown in red.

CHAPTER 3

D-cycloserine (DCS) Activation of N-methyl-D-aspartate Receptors (NMDAR) 24 & 48hr After Asphyxial Cardiac Arrest (ACA) Has No Effect on Hippocampal CA1 Neurological Deficits[1]

3.1. Abstract

Cardiac arrest (CA) results in severe neurological impairments and death through excitotoxicity, especially via *hyperactivation* of *N*-methyl-D-aspartate receptors (NMDAR). P*artial* NMDAR activation, however, can actually restore hippocampal synaptic plasticity by strengthening glutamatergic synapses, thus improving spatial learning and memory. We hypothesized that NMDAR channels were down-regulated from hours to days after resuscitation (ROSC, restoration of spontaneous circulation) from CA in *Sprague dawley* male rats (250-330g). Low dose D-cycloserine (DCS) (10mg/kg, IP), a partial NMDAR agonist, was administered in rats 24 and 48hr after 6 or 8-min of asphyxial CA. Heart rate and blood pressure (mean, systolic, and diastolic) all showed a similar decline after 3-4min of CA regardless of duration. Neurological deficit scores (NDS) (at 2hr and daily for 7 days after CA) for 6 or 8-

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[1] Combs, Vélvá M., Crispell, Heather and Drew, Kelly L. (2013). D-cycloserine (DCS) Activation of N-methyl-D-aspartate Receptors (NMDAR) 24 & 48hr After Asphyxial Cardiac Arrest (ACA) Has No Effect on Hippocampal CA1 Neurological Deficits. Prepared for submission to the *Journal of Neurochemistry*.

min CA were significantly higher during the first 3 days of scoring ($P<0.05$, Tukey) and eventually declined near "0". DCS had no effect on NDS. DCS also had no effect on normal CA1 hippocampal cell counts ($P>0.05$, Tukey). Although DCS showed no neurological improvement, further research on the specific NMDAR activation window post-CA injury could potentially offer clinical insight into CA treatment options.

3.2. Introduction

Recovery after cardiac arrest (CA) is generally very poor and usually results in death or severe neurological impairment due to *global cerebral ischemia* (restricted blood flow to the entire brain) and its hypoxic excitotoxic effects. Over 300,000 people annually are affected by CA in the United States, whereby only 13,800 (4.6%) survive, 42,000/70,000 (60%) of patients that are resuscitated die from brain injury, and only 2,100-7,000/70,000 (3-10%) resume a somewhat normal lifestyle (Krause et al., 1986, Hypothermia after Cardiac Arrest Study, 2002, Nichol et al., 2008). Neurological impairments include short-term memory loss (primarily affecting the CA1 hippocampus region of the brain), speech impairments, and motor and cognitive dysfunction. *Hyperactivation* of N-methyl-D-aspartate receptors (NMDAR) plays a major role in excitotoxic neuronal injury and has led to the need to use NMDAR antagonists in order to slow down or stop the rate of excitotoxic neuronal loss (Yaka

et al., 2007). Previous traumatic brain injury (TBI) research held the belief that blockage of NMDAR hyperactivation after injury would decrease neuronal death and improve recovery; however, clinical trials have failed to show any actual benefit of blocking NMDAR (Biegon et al., 2004). There are three main reasons why these clinical trials may have failed in the past: 1) Hyperactivation of glutamate-gated NMDAR only occurs for a very short period of time (<1hr after injury), but its effects on neuronal injury actually last much longer (usually ≥7 days after initial injury), 2) There is a short therapeutic time window (<30min after injury) in which NMDAR antagonists prove to be most efficacious and lose their effectiveness if given after this point, and 3) Re-stimulation of NMDAR after injury may actually contribute to the regeneration of new synapses (improve synaptic plasticity) in order to compensate for the initial loss of synapses due to injury (Biegon et al., 2004). Biegon et al., 2004 has shown that NMDAR are "down-regulated" in the hippocampus as early as 8hr to as late as 7 days after closed head injury (CHI) in mice from its initial hyperactivation 15min within initial injury (Biegon et al., 2004).

Previous literature (Yaka et. al., 2007) has shown that D-cycloserine (DCS), a partial and selective agonist of NMDAR at the glycine site, improved neurological outcome in mice subjected to CHI and stimulated hippocampal LTP (long-term potentiation), a form of synaptic plasticity, in the previously damaged CA1 brain region (Yaka et al., 2007). DCS is thought to play a role in increasing the rate of NMDAR channel opening, which results in higher spatial learning performances

(Pitkanen et al., 1995a). No study (to our knowledge) has yet addressed if NMDAR stimulation from hours to days after *global cerebral ischemia* will improve neurological outcome from asphyxial cardiac arrest (ACA) in rats. Partial activation of NMDAR is hypothesized to improve outcome following global cerebral ischemia caused by ACA.

We hypothesized that NMDAR channels were down-regulated from hours to days after resuscitation (ROSC, restoration of spontaneous circulation) and that re-stimulation of these receptors would improve neurological outcome in male *Sprague dawley* rats (250-330g). Our goal was to improve neurological recovery by restoring NMDAR activity back to baseline levels without over stimulating receptors and initiating another excitotoxic cascade. In this study, we induced global cerebral ischemia in rats via 6 or 8-min of ACA. We resuscitated animals within 2min. after ACA, and then administered a low dose (10mg/kg, intraperitoneally [IP]) of DCS at 24 and 48hr after ROSC. Neurological deficit scores (NDS) were taken 2hr after ROSC and daily during a 7 day period after ROSC in order to assess neurological severity. CA1 hippocampal cell counts were also measured to determine any neuronal cell loss resulting from ischemia. Our results showed no effect of DCS on neurological improvement, but did show a significant effect of increasing CA duration on neurological impairment as indicated by higher NDS during the first three days after ROSC.

3.3. Methods

3.3.1. Animals

All animal procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Alaska Fairbanks (UAF) (**Appendix K**). Male Sprague-Dawley rats were purchased from Simonsen Labs (Gilroy, CA). Female rats were not used for the present study because of possible neuroprotective interference with neurological assessment and/or drug-interaction due to estrous cycles. Rats were group-housed initially prior to quarantine for 1 week and transferred after quarantine to a procedure area where they were housed in pairs and allowed to acclimate for 2-3 weeks prior to use. Rats were housed 21-23°C on a 12:12-hour light/dark cycle. Food and water was available ad libitum prior to experimentation and during recovery (1 week) after cardiac arrest, except that rats were fasted 24hr prior to surgery. Experiments consisted of four groups of animals where 6 or 8-min ACA was followed by coded treatment with D-cycloserine (DCS) or vehicle (saline). A group of naïve animals were also included for comparison. All rats used for this study weighed between 250-330g.

3.3.2. Cardiac Arrest Procedure

All animal experiments were conducted from November 2008 through December 2009. The surgical procedure was performed as described previously (Katz et al., 1995; Dave et al., 2006; **Ch. 2**). Animals were induced with 5% isoflurane and a 30:70 mixture of oxygen and nitrous oxide followed by endotracheal intubation. Anesthesia was maintained between 1-2% isoflurane. Head and rectal temperatures were maintained between 36.5-37.5°C using temperature controlled heating lamps and Omega (Stamford, CT) T-CSC32 temperature controllers throughout the procedure and for 60min post-ROSC. A three lead electrocardiogram (ECG) was attached to the fore and hind limbs to monitor heart rate. The femoral vein was cannulated using a single lumen (Tygon® flexible tubing; Saint-Gobain PPL Corp., 0.375-mm ID, 0.75-mm OD; length 0.029" & wall 0.014"; Norton, Akron, OH; AAQ041889; Lot No. 112046) catheter and advanced ~ 2 cm towards the heart. The femoral artery was cannulated using the same type of tubing for continuous blood pressure monitoring and blood gas analysis. After cannulation vecuronium (0.3mL, approximately 1 mg/kg) (Gensia Sicor pharmaceuticals, Irvine, CA) was injected intravenously followed by mechanical ventilation (60 breaths per min or bpm) and isoflurane was lowered to 0.5%.

Blood gases were continually sampled (with at least 10min intervals between sampling) until physiological levels were normalized for CA induction. Arterial blood gases, including, Pco_2, Po_2, pH, HCO_3^- and ABE were analyzed (iSTAT

analyzer with GC8+ cartridges; Abbott Laboratories, Abbott Park, IL) and maintained within normal limits by adjusting respiratory rate (60rpm) and tidal volume (stroke volume 2.0mL-3.2mL/min). MABP and ECG were also continuously monitored using an AMP 6600 blood pressure amplifier, and an AMP 6600 bioelectric amplifier (Gould Instrument Systems, Valley View, OH).

Asphyxial CA was induced by disconnecting the ventilator from the endotracheal tube for 6 or 8-min of asphyxiation. Positive End Expiratory Pressure ("PEEP"; 5cm column of water) was implemented during CPR. Resuscitation was attempted for 2min or less following epinephrine (10ug/kg, i.v.) injection followed immediately by bicarbonate ($NaCHO_3$, 0.9cc, i.v.) injection to reduce acidosis. Ten minutes after ROSC, blood gases were sampled again. Immediately following blood gas sampling, ventilator rate was decreased from 80 to 60 rpm and oxygen lowered from 100% to 30% in a mixture with N_2O. IPTT-300 temperature transponder tags (Biomedic Data Systems [BMDS] Inc., Seaford, DE) were implanted subcutaneously [SC] at the end of surgery.

3.3.3. Post-Operative Care

Post-operative (post-op) rats were placed in a neonatal incubator set to 29.0°C overnight with water provided. Saline (0.9%, IP) was administered in a volume of 1mL/100g body weight at the end of surgery to prevent dehydration. After 8-16hr

animals were fed, weighed, cleaned, and assessed for neurological deficit scores (NDS). This was repeated daily for 7 days (Note: NDS were also assessed 2hr post-ROSC). Rats were fed a 50:50 mix of rodent chow and sugar mixed with water to create a liquid "soupy" chow mixture which was placed in small petri dishes to allow animals to self-feed. Animals that were unable to feed themselves were assisted via "spoon-feeding". A gavage needle (≈ 11 cm long, 16ga x 1 ½", blunt-ended, with bulb diameter of 4.0 mm) and a 3cc syringe were used to deliver liquid soup material into the animal's mouth. Animals were fed until they no longer swallowed in response to food delivery into the mouth. Most rats were spoon-fed and consumed the entire 3cc volume. Rats would feed themselves by consuming dry rodent chow on subsequent days. Wounds were cleaned once per day with dilute betadine to prevent risk of infection. Exclusion criteria: only animals resuscitated within 2 minutes post-ACA were included in data analyses.

3.3.4. Drug Administration

D-cycloserine (DCS) was purchased from Sigma-Aldrich (St. Louis, MO; C6880-1G) and dissolved in saline and delivered intraperitoneally (IP) at 10mg/kg in a volume of 1.0mL/kg. This DCS dose improved acquisition of spatial memory tasks without inducing concurrent side effects (Lelong et al., 2001). Vehicle consisted of sterile saline (0.9% NaCl, 1.0mL/kg, IP). DCS and Vehicle were sterilized by

filtering through a 0.2µm filter (Pall Corporation, Ann Arbor, MI; PN 4192) into a sterile red top vacutainer test tube. Both solutions were freshly prepared on the first day of injection (24hr after resuscitation) and given to rats 24 and 48hr after CA. DCS or vehicle was administered and NDS assessed by an observer unaware of treatment. Adverse DCS symptoms were not seen nor reported in previous literature (Yaka et al., 2007).

3.3.5. Neurological Deficit Scoring Assessment

Neurological deficit scores (NDS) were taken at 2hr after ROSC and daily for 7 days after CA (see **Appendix E** for NDS worksheet). Total NDS assessed five components: consciousness and respiration, cranial nerve function, motor function, sensory function and coordination (Katz et al., 1995). The NDS scale ranged between "0" (no neurological deficiency; normal function) to "100" (maximum neurological deficiency). To maintain consistency between NDS animal results, NDS was performed by a single observer. NDS was collected through day 7 until animals were euthanized and brains collected for histopathology 8 days after ROSC.

3.3.6. Histology

On day 8 following ROSC, rats were perfused with FAM solution (100mL of 36% formaldehyde, 100mL 99.5% glacial acetic acid, and 800mL 99.8% methanol per 1L solution; 1:1:8 by volume) for 19min after a 1-min initial perfusion with physiological saline (see **Appendix G**). The perfusate is delivered at a constant rate ≈ 80mL/min of into the left ventricle of the heart (**Ch. 2.3**) with the descending aorta clamped. The rat was decapitated and the head was immersed in FAM at 4°C for 24hr. After an initial RO-H_2O wash for 10-15min, brains were carefully removed from the skull and stored in fresh FAM for another 24hr at 4°C. Finally, brains were placed in 70% ethanol solution until trimmed and sectioned into coronal brain blocks for paraffin embedding. Coronal sections of 8μm were stained with hematoxylin and eosin (see **Appendix H, I**). Rat brain sections examined contained the hippocampus at approximately-3.8 mm posterior to bregma. Normal ("healthy", *non-pyknotic*) neuronal counts were made within a section of the CA1 region of hippocampus by an investigator blinded to the experimental conditions and the counts are expressed as number of normal neurons present per mm of CA1 (at 40X magnification). Normal neurons were defined as having a well-defined cellular membrane and distinct cellular nucleus. MetaMorph 7.0 software was used to record cell counts.

3.3.7. Statistics

Data are expressed as mean ± SD with the exception of NDS data which is expressed as median and quartile values. Statistical evaluation was performed using MANOVA with repeated-measures over time followed by a Tukey multiple-comparison *post-hoc* test used to measure differences between group means (SAS v9.1 software; SAS Institute Inc., Cary, NC, USA) or *t-test* where indicated; $P < 0.05$ was considered significant. ANOVA single factor statistical test was used for CA1 hippocampal cell counts. Five rats were excluded from data analysis which included rats not surviving to 48hr post-ROSC (n=1), had poor brain tissue fixation (n=3), or did not have hippocampal brain tissue trimmed -3.80mm from bregma (n=1).

3.4. Results

3.4.1. Physiology

Physiological parameters (*age*, *body weight*, Po_2, Pco_2, *pH*, *acid base excess (ABE), hematocrit (HCT), plasma glucose*, HCO_3^-, *mean arterial blood pressure [MABP], and temporalis/rectal temperatures*) were monitored before as well as after CA to explore if experimental groups differed before or after treatments. Age, body weight, HCT, and plasma glucose (mg/dL) did not differ between experimental animal groups. pH, HCO_3^-, and ABE all significantly increased after CA while

MABP decreased from baseline values after CA (**Table 3.1**; P<0.05, MANOVA, main effect of time for each variable). MABP values overall were lower in 8-min than in 6-min rats (**Table 3.1;** P=0.0016, MANOVA main effect of duration). 8-min CA rats had significantly lower Po_2 than 6-min both before and after CA regardless of drug treatment (P=0.0294, MANOVA, main effect of duration). T_{brain} and T_{rec} values were overall slightly higher in 8-min rats than 6-min CA rats which had lower T_{brain} values, but all within the 36.5-37.5°C range (**Table 3.1**; P=0.0220 [T_{brain}], P=0.00033 [T_{rec}]; MANOVA, main effect of duration). T_{rec} values in all groups were slightly higher "After CA", but were all within 36.5-37.5°C (**Table 3.1**; P=0.0141, main effect of time). There were no differences between physiological parameters prior to drug treatment.

3.4.2. Asphyxial CA

Heart rate (HR) and blood pressure (MABP, systolic [SBP], and [DBP]) were compared before, during, and after CA between experimental groups. For the most part groups showed similar severity of CA with declines in heart rate and blood pressure over time (P<0.0001 for HR, P<0.0001 for MABP, P<0.0001 SBP; P<0.0001 DBP; MANOVA, main effects of time). However, for heart rate, MABP, SBP, and DBP there were some statistical differences between 6 and 8-min CA groups at some time points (**Fig. 3.1**) (For HR, P<0.0001, MANOVA, time × duration

of CA; for MABP P=0.0030, main effect of duration, P<0.001 time × duration of CA; for SBP P=0.0035, main effect of duration; P=0.0016, time × duration; for DBP, P=0.0048, main effect of duration; P=0.0021, time × duration). No differences in heart rate or blood pressure were seen in groups subsequently treated with DCS or saline (**Fig. 3.2**). Immediately after induction of asphyxiation, bradycardia (~ ≥60% HR reduction) and hypotension (<50mmHg) was observed in all animals starting around 2min of CA. For 6 or 8-min CA rats, HR and MABP slightly decreased within 1min of CA and significantly decreased within 2-4min of CA with values near 0 mmHg by 4min of CA (see **Fig. 3.1**). CA duration time intervals of "Before CA" (2min prior to CA induction), "1-2min", and "2-3min" significantly differed between both 6-min and 8-min CA rat groups (P<0.05, Tukey). DCS and vehicle saline groups had similar declines in HR and MABP and systolic and diastolic blood pressures during CA (**Fig. 3.2**, P>0.05).

3.4.3. Neurological Deficit Scores (NDS)

To assess the extent of injury caused by CA or therapeutic effects of DCS we compared NDS between experimental groups. NDS scores were not normally distributed, however, transformations failed to improve normal distribution of residuals. Therefore data were analyzed using ANOVA and are shown as median and the first and third quartiles. Note that when data are not normally distributed medians

and quartiles are a more accurate measurement of 'central tendency' than mean ± SEM. CA produced significant neurological deficit (indicated by higher NDS values) as shown in Total NDS that spontaneously recovered over time (a majority of rats eventually had a NDS of "0" or "near 0" by the end of the 7-day assessment) ($P<0.0001$, MANOVA, main effect of time; **Fig. 3.3**). Rats exposed to 8-min CA showed greater and prolonged injury (especially during the first three days of scoring) which slowed down recovery time as opposed to 6-min CA rats which had faster recovery times and less neuronal injury (**Fig. 3.33a, b**; $P=0.0026$, main effect of duration; $P=0.0003$, time × duration). DCS treatment had no effect on Total NDS (**Fig. 3.3c, d**). Categorical NDS was also analyzed within the following categories: *General Behavioral, Cranial Nerve Reflexes, Motor, Sensory*, and *Coordination*. All categorical NDS showed significant differences between 6 and 8-min CA rats over time (NDS Days 1-3) ($P<0.0001$, main effect of time; **Fig. 3.4**). 8-min CA also produced significant injury in rats for both Total and Categorical NDS (Motor NDS $P=0.0039$, main effect of duration; $P=0.0058$, time × duration; **Fig. 3.4a**; Sensory NDS $P=0.0008$ main effect of duration; $P=0.0004$, time × duration; **Fig. 3.4b**; and Coordination NDS $P=0.0023$, main effect of duration; $P=0.0003$, time × duration; **Fig. 3.4c**; General $P=0.0242$, main effect of duration; $P=0.0021$, time × duration; **Fig. 3.4d**; Cranial $P=0.0167$, time × duration; **Fig. 3.4e**) which contributed to slower recovery. Overall, DCS did not produce a significant effect on Total or Categorical (Motor, Coordination, and Sensory) NDS; slight differences between DCS and saline rats were seen for General (NDS Day 5, $P<0.05$, Tukey; **Fig. 3.5a**) and Cranial (NDS

Days 5 & 6, $P<0.05$, Tukey; **Fig. 3.5b**) NDS, respectively. DCS has no significant effect on NDS.

3.4.4. Histopathology of CA1 Hippocampus

All CA treated rats showed a significant reduction in the number of healthy CA1 neurons in the hippocampus when compared with naïve rats; (**Fig. 3.6**). Mean ± SD for CA and naïve rats were as follows: 180.8 ± 27.6 naïve (n=5) vs. 46.3 ± 33.8 [all CA groups, n=27]; ($P=7.12\times10^{-5}$, two-sample unequal variances *t*-test). There was no significant differences seen in CA1 neuron counts for 6 and 8-min CA rats and DCS had no significant effect on cell counts (**Fig. 3.6**). Ischemic neurons (shrunken "pyknotic" cells with darkened triangular-shaped nuclei, and eosinophilic nucleolus and cytoplasm) were also present in the CA1 region of all CA treated rats; especially in 8-min CA DCS rats, but were not counted for histopathological analysis (**Fig. 3.7**).

3.5. Discussion

The aim of this study was to determine if activation of down-regulated NMDAR function by DCS after ischemic injury could improve neuronal outcome subsequent to asphyxial CA. Our results showed that DCS had no significant effect

on neurological improvement from asphyxial CA in either 6 or 8-min CA rats as assessed by NDS and histopathology. These results fail to support our initial hypothesis that DCS would improve neurological outcome after CA. Our NDS results showed greater injury and slower recovery time for 8-min CA rats while histopathology results showed a severe reduction of healthy hippocampal CA1 neurons in CA rats compared to naïve rats, in rats subject to 6 or 8-min of CA. These results suggest that 6-min CA injures the ischemic vulnerable CA1 region to a maximal degree.

DCS ineffectiveness in improving brain injury could be attributed to four possible main factors. First, DCS treatment possibly did not coincide with NMDAR down-regulation. NMDAR down-regulation only occurs for a limited period of time (Bickler and Buck, 1998, Biegon et al., 2004) and DCS could have been administered at a time point in which its efficacy may have no longer proved to be effective. It has been hypothesized that long-term suppression of NMDAR function exacerbates progressive brain injury following cardiac arrest as described for traumatic brain injury (Yaka et al., 2007), however NMDAR down-regulation after CA has not been studied. In fact, the exact duration of NMDAR hyperactivation (generally, <1hr) post ischemic/TBI injury still remains unknown; NMDAR hypofunction occurs a few hours after injury and lasts for 24hr or more (≥7days) (Biegon et al., 2004). Second, the severity of injury may have been too great to see improvement. 6-min CA may have already produced maximal neuronal injury and any attempt of alleviating injury

at this point (or at 8-min CA) would not be seen. This possibility is supported by the observation that 6 and 8 min of CA produced the same decrease in the number of healthy neurons in the CA1 region suggesting that a maximal degree of CA1 injury was sustained by 6 min of CA. Third, DCS drug dosage could have been too low or too high to show any therapeutic effect. DCS dosage was chosen based on improved outcome following TBI in mice (Yaka et al., 2007); however, the dose-response relationship may depend on the time course of NMDAR down-regulation as well as the species and severity of brain injury. At high doses, DCS generally produces an antagonistic effect which competes with other agonists for the same NMDAR binding sites while at moderate doses, DCS produces a partial agonistic effect which facilitates glutamatergic NMDAR transmission (Horio et al., 2012). Fourth, histopathology and NDS assessment may not have been sensitive enough to detect more subtle differences in hippocampal function and histopathology did not allow for study of the time course of hippocampal injury. Measure of hippocampal dependent cognitive dysfunction (e.g. Morris water maze, radial arm maze, contextual fear conditioning, or fear-extinction conditioning behavioral tests) could be used to assess the time course of CA1 hippocampal deficiency (Kesslak et al., 1998, Hall et al., 2000, Mizuno et al., 2000, Cunha et al., 2010). Poor recovery from CA is now understood to be attributed to progressive neuronal death over a period of days to months after injury so assessment of the time course of hippocampal function is warranted (Dave et al., 2006).

Physiological parameters were consistent among DCS and saline treated groups so it is unlikely that a difference in the severity of CA confounded the effects of DCS. pH, HCO_3^-, and ABE all increased significantly after CA probably in response to the HCO_3^- flush which occurs immediately after successful resuscitation in order to control for acidosis. Longer CA duration caused significantly lower Po_2 and MABP. These differences are consistent with what is expected from longer duration of CA and with the worse outcome observed after the longer duration of CA. CA generally results in lower pH in tissues, arterial hypotension, and hypercapnia clinically in prenatal, pediatric, and some adult populations (Katz et al., 2002). The asphyxial CA rat model produced neuronal injury that is consistent with previous CA literature (Katz et al., 1995, Dave et al., 2006) by which HR and BP decreased within 3-4 min of CA onset. Results show statistically different declines in HR and BP over time in 6 and 8-min CA rats. The decline in BP was slightly faster in rats subsequently exposed to 8-min of asphyxia. No differences were seen between DCS and saline (both declined at similar rates). Another important factor to note is the elevated T_{brain} in 8-min CA rats. Slower recovery time in 8-min CA rats could have been attributed to overall higher T_{brain}, or to slightly more severe decreases in BP during 1-3min of asphyxia.

It was somewhat unexpected for DCS to not have any effect, especially in histopathology analyses. Previous studies have shown that DCS improves recovery after closed head injury (Yaka et al., 2007) and that DCS facilitates changes in

hippocampus-and-amygdala dependent learning (Monahan et al., 1989, Flood et al., 1992, Thompson et al., 1992, Kuriyama et al., 2011). Past human preclinical DCS trials have even shown elevation of activity in the CA1 region of the hippocampus compared to placebo groups (Grunwald et al., 1999). This effect occurs indirectly by increasing the learning efficiency through which upregulation of glutamate signaling in NMDAR above normal threshold levels occurs (Onur et al., 2010). In the brain, DCS has a higher selective affinity for the glycine site of NMDAR where granule cells are potentiated; DCS stimulates NMDAR-mediated transmission by freely entering into the blood brain barrier (BBB) and has been shown to enhance field potentials in the hippocampus, typically seen in highly concentrated NMDAR areas of the brain like the CA1 region of dorsal hippocampus and the dentate gyrus (Pitkanen et al., 1995b).

The main caveat regarding this study is that the exact NMDAR down-regulation time course remains unknown for the ACA injury model. Another caveat is that the mechanism for neuroprotection via DCS in TBI remains unclear. Other studies have shown that restoration of NMDAR channel activity increases *brain-derived neurotropic growth factors (BDNF)* levels, which can cause a positive feedback loop effect to further enhance hippocampal function. In fact, the hippocampus has the highest levels of BDNF mRNA and BDNF gene receptor, *trk*B, which play a vital role in synaptic plasticity (Hofer et al., 1990, Klein et al., 1990, Ma et al., 1998). One big disadvantage to this approach, however, is that there is a critical time window

involved whereby restoring NMDAR back to normal levels is only beneficial in the subacute post-injury phase. Also, overstimulation of NMDAR can cause the same excitotoxic effects that were originally initiated by the ischemic injury. Thus, more work is needed before dismissing the potential for a NMDAR partial agonist to improve outcome after global cerebral ischemia.

3.6. Conclusion

DCS had no significant effect on neurological improvement following asphyxial CA. Asphyxial CA duration (6 and 8-min) played a significantly larger role in affecting neurological outcome as expressed in NDS and CA1 neuron counts than DCS treatment. Additional research is needed to further evaluate the therapeutic effectiveness of NMDAR activation after excitotoxic ischemic injury in animal models when NMDAR are expected to be down-regulated.

3.7. Acknowledgments

This study was supported by National Institutes of Health grant NS41069-06, US Army Medical Research and Materiel Command (# 05178001), UAF Center for Research Services, and Flint-Hills Resources. A special thanks to our University of Miami Miller School of Medicine colleagues Dr. Kunjan R. Dave and Isabel Saul for

technical assistance and troubleshooting; Dr. June Sun, Jeanette Moore, Dr. Tulasi Ram Jinka, Kimberly E. Iceman, and Zachary Carlson for initial CA setup and surgical/post-operative assistance; and Dr. Julie McIntyre for statistical analysis assistance.

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Table 3.1. Physiological parameters measured before and after asphyxial cardiac arrest (CA) in rats.

Physiological Parameters for 6 min CA rats.

		Ischemia (6 min)			
		Before	After	Before	After
Group	Variable	D-cycloserine (DCS)		Vehicle (Saline)	
DCS Rats (n = 10)	Age (days)	68.9 ± 4.3		68.5 ± 5.5	
Vehicle Rats (n = 8)	Body Weight (g)	284.7 ± 10.7		283.7 ± 17.6	
	pH	7.46 ± 0.03	7.50 ± 0.07	7.44 ± 0.02	7.46 ± 0.02
	P_{CO2} (mm Hg)	38.0 ± 2.3	37.8 ± 2.2	38.8 ± 1.7	41.1 ± 3.0
	P_{O2} (mm Hg)	119 ± 7.1	135 ± 44	122 ± 7.3	128 ± 14
	HCO_3^- (mmol/L)	27.3 ± 1.8	29.8 ± 4.2	26.2 ± 1.5	29.1 ± 2.5
	Base Excess	3.5 ± 2.1	6.7 ± 5.3	2.1 ± 1.6	5.3 ± 2.6
	HCT	48.2 ± 2.0		48.5 ± 1.5	
	MABP (mm Hg)	120 ± 16	96 ± 14	116 ± 9.5	93 ± 17
	T_{brain} (°C)	36.6 ± 0.2	36.7 ± 0.2	36.7 ± 0.2	36.6 ± 0.4
	T_{rec} (°C)	36.8 ± 0.2	37.0 ± 0.3	36.8 ± 0.2	36.8 ± 0.5
	Plasma glucose (mg/dL)	144 ± 16		143 ± 22	

Note: One 6-min CA rat is missing "After" ischemia physiological values for all variables.

Physiological Parameters for 8 min CA rats.

		Ischemia (8 min)			
		Before	After	Before	After
Group	Variable	D-cycloserine (DCS)		Vehicle (Saline)	
DCS Rats (n = 7)	Age (days)	74.0 ± 6.4		68.8 ± 6.1	
Vehicle Rats (n = 6)	Body Weight (g)	299.2 ± 24.3		275.4 ± 16.5	
	pH	7.45 ± 0.01	7.48 ± 0.04	7.44 ± 0.03	7.50 ± 0.0
	P_{CO2} (mm Hg)	37.9 ± 1.1	38.1 ± 1.8	38.3 ± 2.7	38.1 ± 2.0
	P_{O2} (mm Hg)	112 ± 10.0	116 ± 34.1	119 ± 10	102 ± 2.5
	HCO_3^- (mmol/L)	26.3 ± 1.2	28.8 ± 2.9	25.8 ± 1.3	30.0 ± 2.1
	Base Excess	2.1 ± 1.3	5.1 ± 3.6	1.7 ± 1.5	6.8 ± 2.4
	HCT	47.8 ± 3.5		46.6 ± 0.07	
	MABP (mm Hg)	109 ± 10.9	72.4 ± 10.5	106 ± 30	74 ± 17
	T_{brain} (°C)	36.9 ± 0.3	36.9 ± 0.4	36.8 ± 0.3	36.8 ± 0.2
	T_{rec} (°C)	36.9 ± 0.4	37.1 ± 0.3	37.0 ± 0.1	37.4 ± 0.4
	Plasma glucose (mg/dL)	144 ± 24		124 ± 16	

All data are expressed as **mean ± SD**. N = 30-31 rats total.

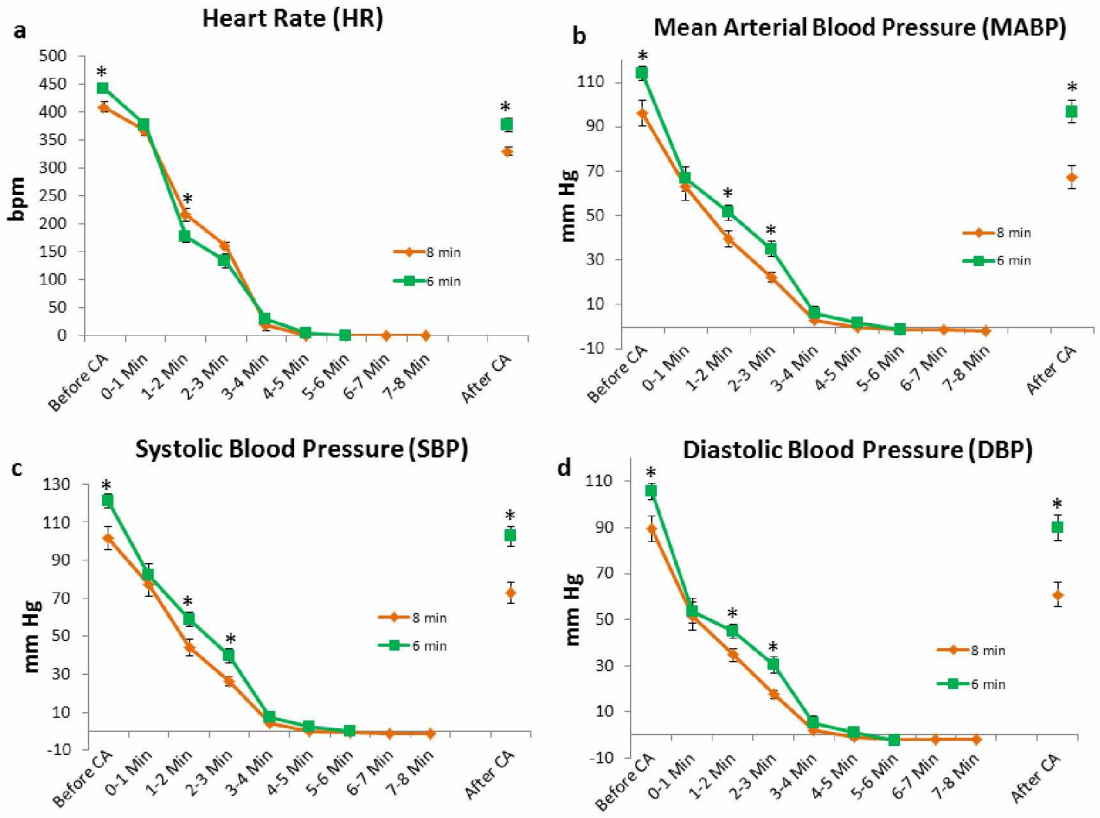


Figure 3.1. LabScribe Values for (**a**) heart rate (HR), (**b**) mean arterial blood pressure (MABP), (**c**) systolic blood pressure (SBP), & (**d**) diastolic blood pressure (DBP) before, during, and after induction of asphyxial cardiac arrest (6 or 8-min CA). The decrease in BP was slightly faster in rats subjected to 8-min CA; *P<0.05, Tukey.

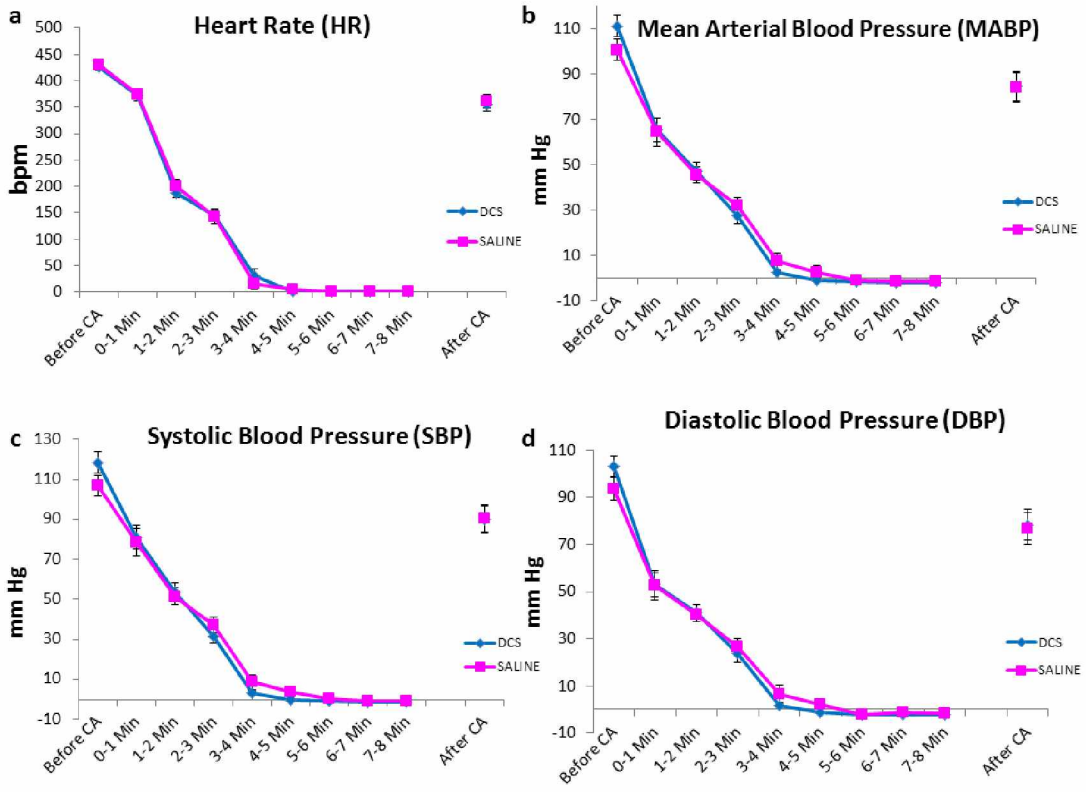


Figure 3.2. LabScribe Values for heart rate (HR) (**a**), mean arterial blood pressure (MABP) (**b**), systolic blood pressure (SBP) (**c**), & diastolic blood pressure (DBP) (**d**) before, during, and after induction of asphyxial cardiac arrest (6 or 8-min) in control (vehicle saline) or experimental (D-cycloserine, DCS) rats. Drug was administered IP in rats 24 & 48hr post-resuscitation.

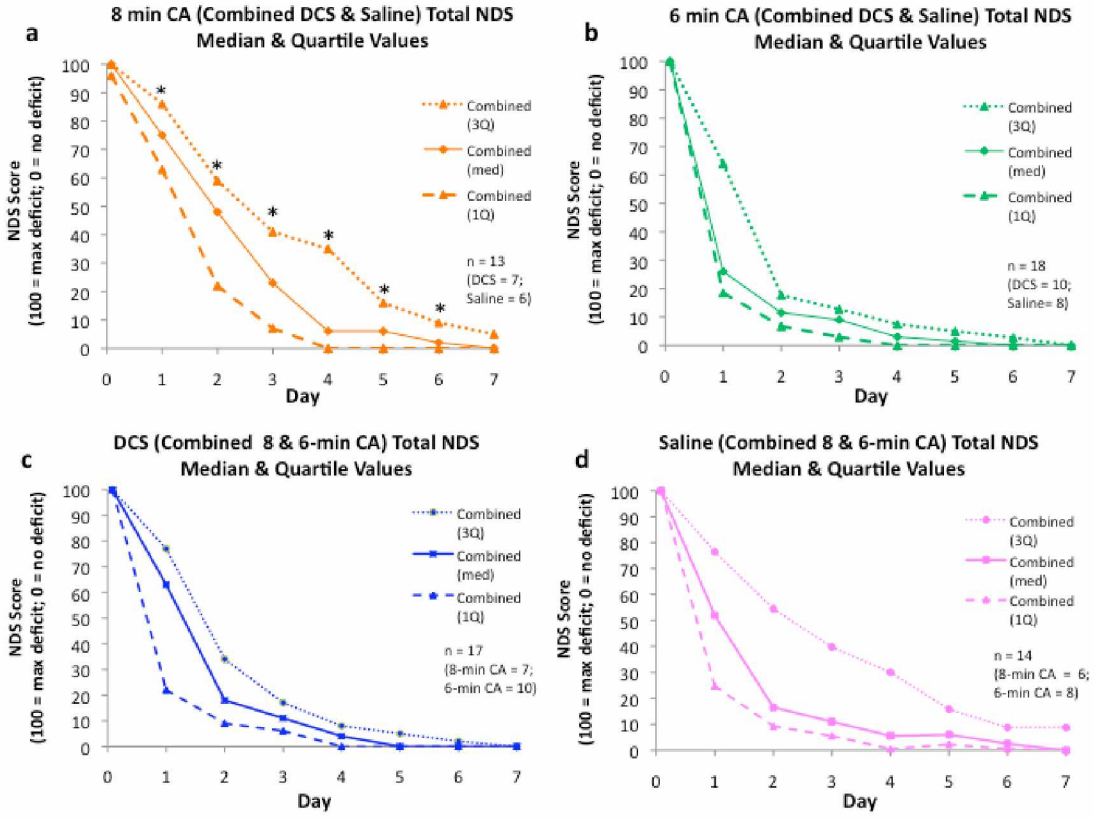


Figure 3.3. Total Neurological Deficit Scores (NDS) shown for 8-min cardiac arrest (CA) (**a**), 6-min CA (**b**), D-cycloserine (DCS) (**c**), & Vehicle (Saline) (**d**) treated rats. NDS scores were significantly higher during the first 3 days of testing in both 6 and 8-min CA rats and eventually decreased to near 0 over time; however, there were no significant differences observed in NDS scores for DCS and saline treated rats. *P<0.05, Tukey, 6 vs. 8-min, n=6-10.

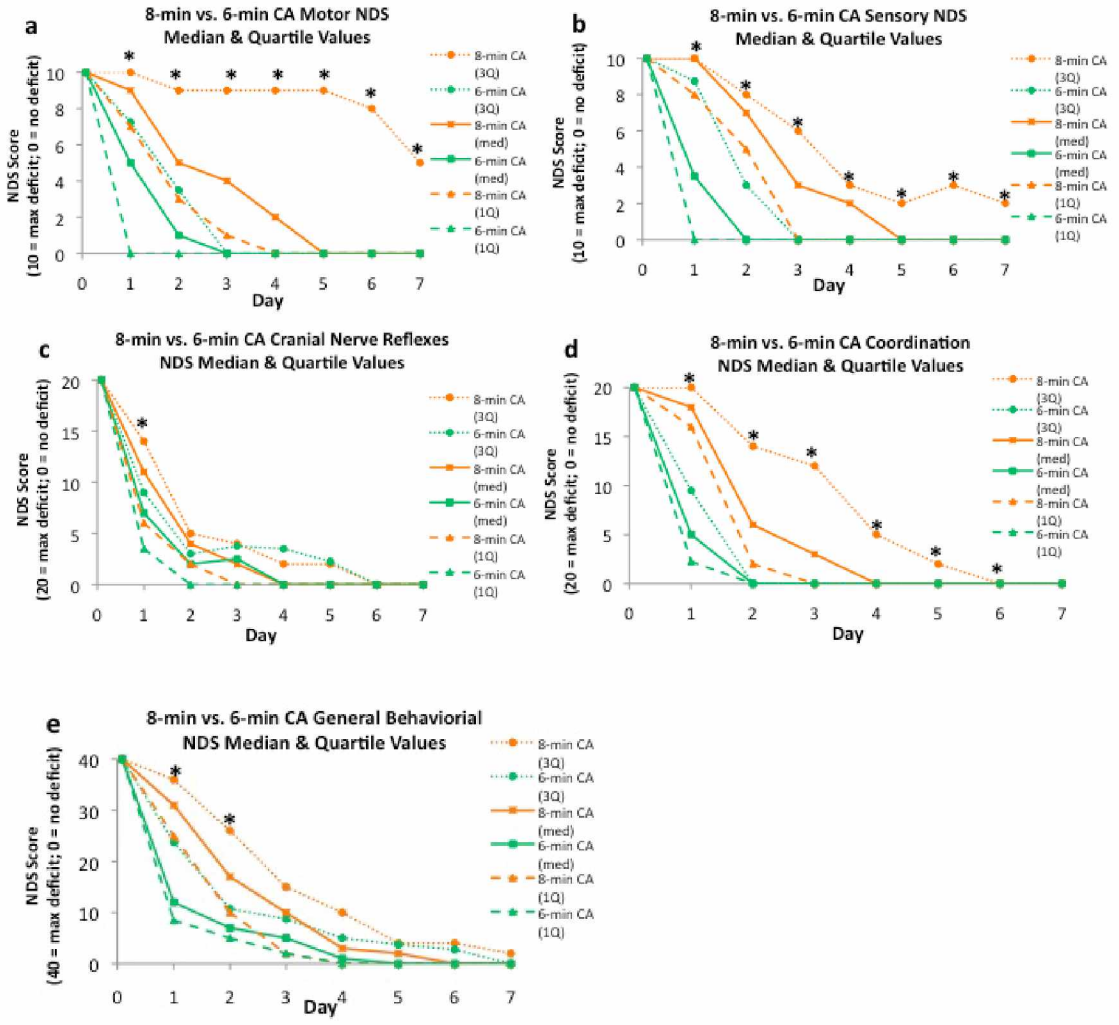


Figure 3.4. Categorical Neurological Deficit Scores (NDS) consisting of **(a)** motor, **(b)** sensory, **(c)** coordination, **(d)** general, and **(e)** cranial components are shown for both 6-min and 8-min cardiac arrest (CA) rats. NDS scores were significantly decreased in both 6 and 8-min CA rats with respect to recovery time. *P<0.05, 6 vs. 8-min, n=6-10.

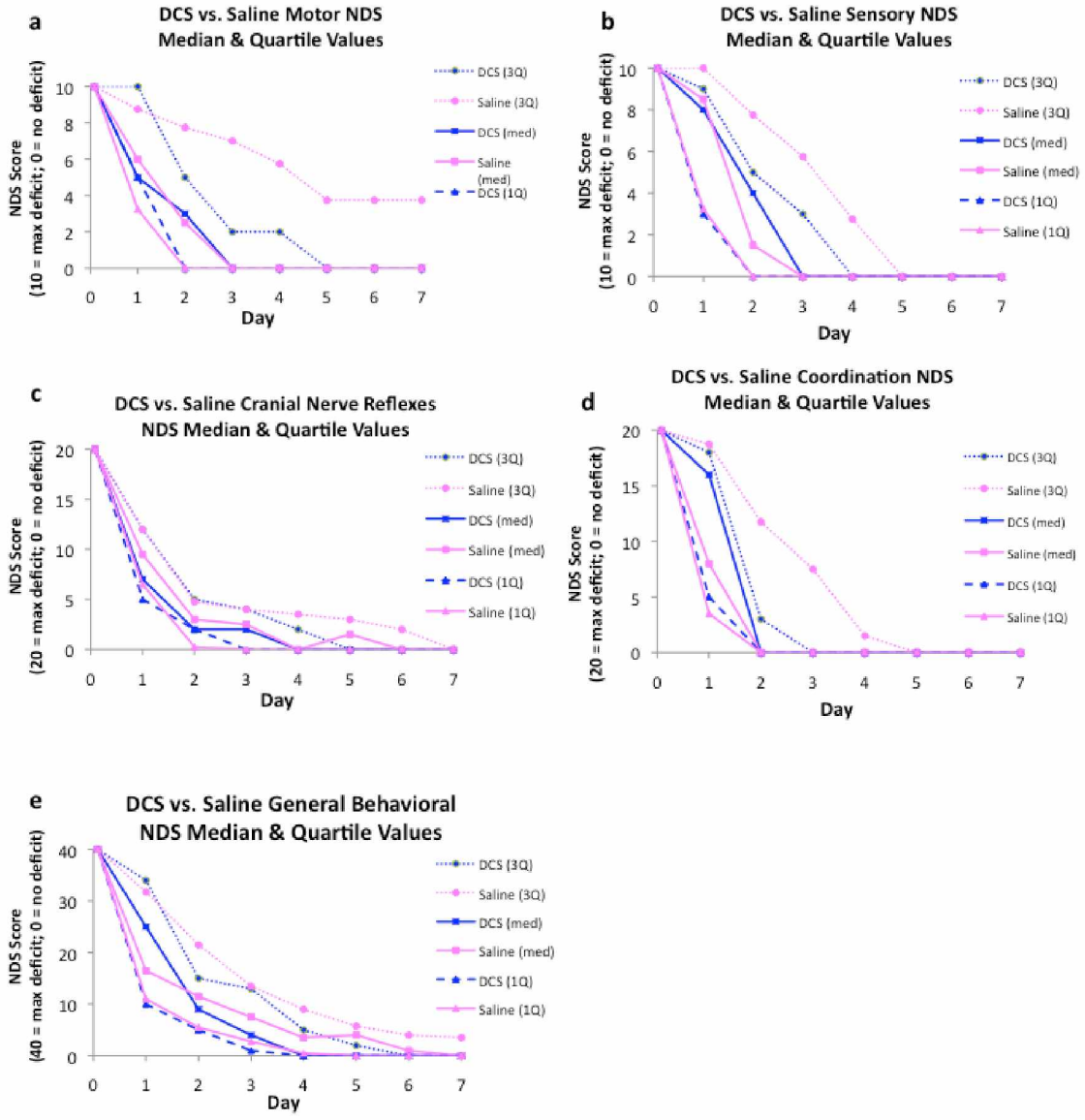


Figure 3.5. Categorical Neurological Deficit Scores (NDS) consisting of (**a**) motor, (**b**) sensory, (**c**) cranial, (**d**) coordination, and (**e**) general components are shown for both DCS and saline cardiac arrest (CA) rats.

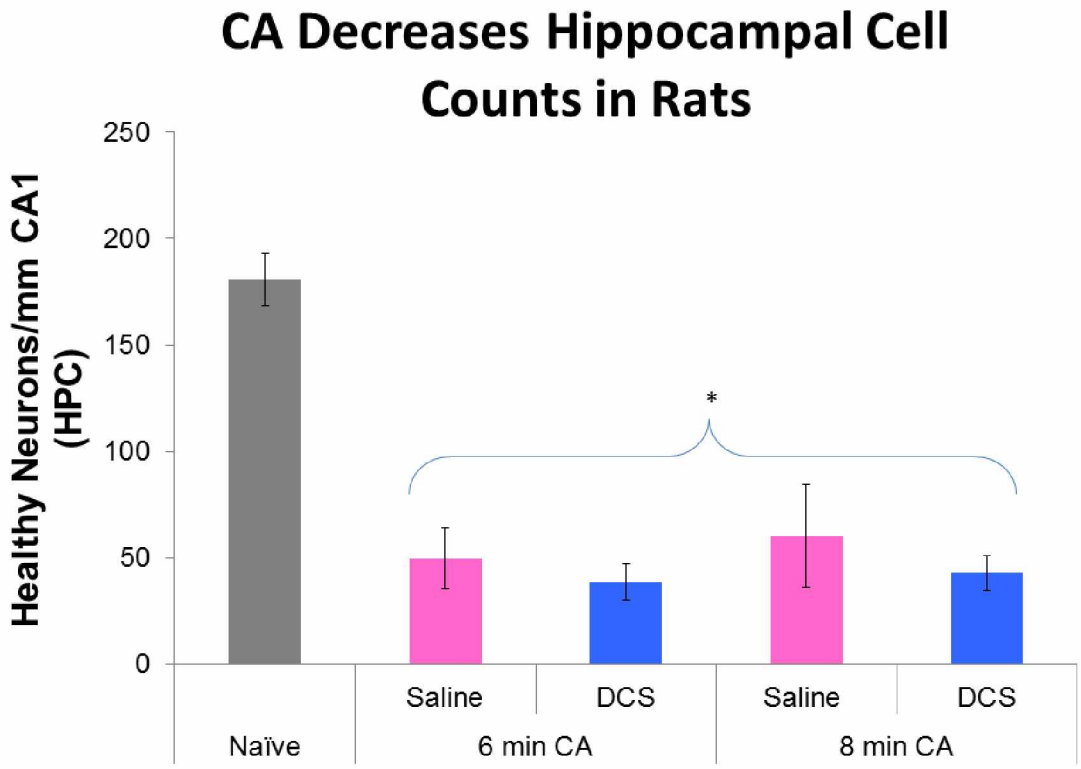


Figure 3.6. Number of healthy normal neurons per mm of CA1 hippocampal (HPC) region in CA duration (6-min CA or 8-min CA) and experimental drug (control: Vehicle Saline; experimental: D-cycloserine, DCS) treated rats 8 days post-ischemic injury and resuscitation. No significant differences were seen for CA1 neuronal cell counts in either 6 or 8-min CA and DCS or saline rats, but all CA groups differed from naïve rats. *P<0.05 CA vs. naïve, n=5-10, t-test.

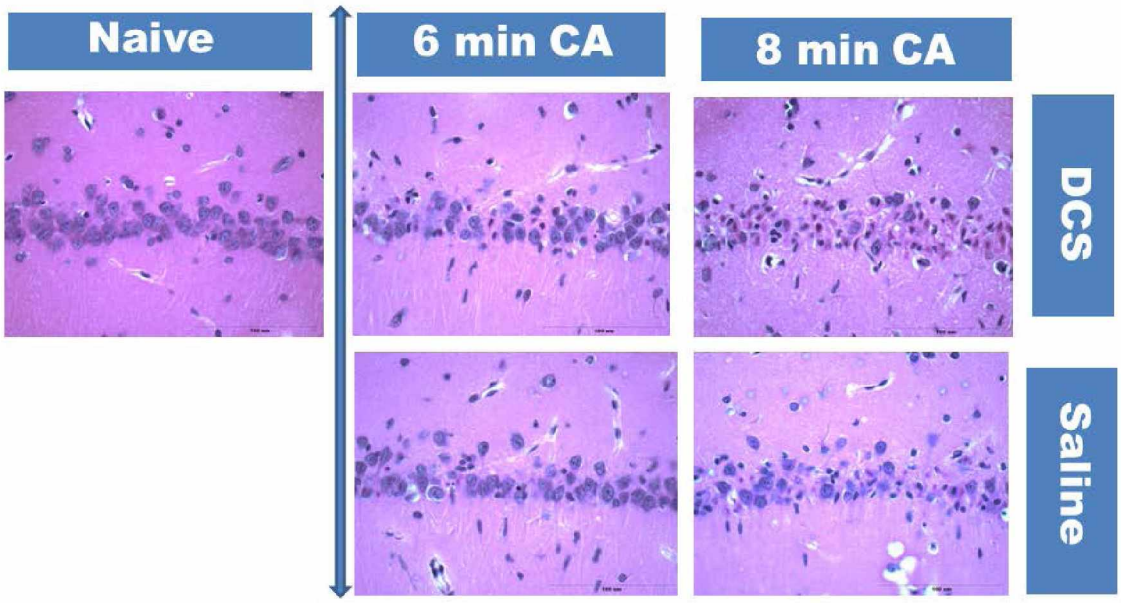


Figure 3.7. Representative images (at 40X magnification) showing histopathology of pyknotic (ischemic) and healthy neurons in the CA1 hippocampal region of drug (D-cycloserine, DCS, or Vehicle, saline) treated rats 8 days after 6 or 8-min asphyxial CA. Scale bar = 100μm.

CHAPTER 4

General Conclusion

The goal of this thesis was to transfer the clinically relevant asphyxial cardiac arrest (ACA) rat model technique already established at the University of Miami (UM) into the laboratory setting at the University of Alaska Fairbanks (UAF). I also used the model to test the hypothesis that stimulation of NMDAR hours-to-days after cardiac arrest would improve outcome. Since NMDAR (N-methyl-D-aspartate receptor) channels are believed to be down-regulated within hours-to-days immediately following reperfusion, I hypothesized that re-stimulation of NMDAR channels back to (or similar to) baseline levels prior to ischemic injury could possibly lead to neuronal improvement. Therefore, I predicted that D-cycloserine (DCS) administration would lead to neuronal improvement by re-stimulating down-regulated NMDAR channels back to (or at least similar to) baseline levels prior to ischemic injury.

Results showed that administration of DCS 24 and 48hr after ROSC (restoration of spontaneous circulation; resuscitation) did not improve neurological outcome in male *Sprague dawley* rats subjected to either 6 or 8-min CA as based on 1 week Neurological Deficit Scoring (NDS) and neurohistopathology (healthy CA1 hippocampal neuron counts) assessments. However, results did show that implementation of the ACA model was very effective at reproducing clinically relevant brain damage (as seen in prenatal and pediatric asphyxia) in rats into the lab setting and can help in the development of future neuroprotective therapies for humans, especially regarding prenatal and pediatric

asphyxial CA.

Further investigation into NMDAR down-regulation time window can potentially offer insight into the effectiveness of various therapeutic agents such as NMDAR antagonists and agonists. Studies on DCS efficacy including dosage and therapeutic time window optimization, and insight into how DCS activation of NMDAR provides neuroprotection can provide new treatment options for various cognitive impairments. ACA rat model improvements involve brain region specific quantification methods for assessment of neurohistopathology after brain injury. For example, use of behavioral assays that specifically test hippocampal function, such as the Morris water maze, may provide better brain injury assessment with greater sensitivity for detecting spatial memory deficits. In addition, the ACA model will be used to investigate the neuroprotective effects of mild therapeutic hypothermia.

Appendix A

Surgery Log

SURGERY LOG

Date:_____ ****IACUC#:**_____ **SPECIES**: _____

ID:_____ **SEX**:_____ **WEIGHT**:_____

** An IACUC number from a current Assurance of Animal Care from must be entered in the log.
All procedures must have been approved and the surgeon identified to the IACUC.

Surgeon:_____ **Assistant**:_____
Assistant:_____ **Assistant**:_____

ANESTHESIA:(you must attach anesthesia monitoring forms to the surgery log)

	AGENT	DOSE (mg)	ROUTE	TIME	COMMENTS
Pre-medication:					
Anesthesia:	Anesthesia is induced and maintained with isoflurane mixed with 70/30 N2O/O2 delivered at 1.5 L/min.				

Post-Op medication:

(Post-Op medication includes all antiseptic, antibiotics, analgesics, ECT)

SURGICAL PROCEDURE: (detail surgical site, site preparation, draping, size of incision, scalpel blade size, blunt and/or sharp dissection, describe manipulation and/or implants, describe samples collected including amount, describe closure including suture material, size and knots, detail all complications encountered during procedure, etc.).

START TIME:_____ **END TIME**:_____

Location of surgery: _____

Table and stereotax were wiped with Nolvasan solution. Surgical instruments and screws were autoclaved or gas sterilized and sterilized with Instrucal lubricant milk between animals. Temperature probes were inserted into both the rectum and the left temporalis muscle, and separate heating lamps or heating lamp and circulating water blanket were used to maintain rectal temperature and temporalis muscle temperature at 36.5°C to 37.5°C. Surgical site was prepared by clipping of the hair using a clipper blade and disinfecting of the skin by using alternate scrubs of beginning with betadine and alternating with 70% isopropyl alcohol for 3 times and finishing by painting site with full strength betadine.

Figure A-1. "Surgery Log" form page 1. This form records physiological data from rats during cardiac arrest surgery.

(Steps are copied from Drew lab cardiac arrest protocol; set up for CA revised October 23, 2009 with changes implemented):

	Notes
1. **Open pressure gauge pack and set up pressure transducer.**	
2. **Prepare injectables** (see pg. 10) **and fill two cannulae w/hep-saline** w/1cc syringe and 23 ga. luer stub adapter (Becton Dickinson; reorder # 427565) for PE-50; 26ga luer stub for Tygon. Fill one 10cc syringe w/sterile saline. Leave sterile ends in pack.	
3. **Anesthetize** with 5% isoflurane and a 30:70 mixture of O_2 (400-500mL/min) and N_2O (1L/min) via scavenger fluovac mask. This insures stable physiological levels for PO_2, PCO_2 and pH.	
4. **Begin endotracheal intubation first if using rats (skip this step & step 5 if using AGS; see step 10 for AGS intubation):** Open sterile intubation pack. First take animal off anesthesia mask, insert otoscope (with speculum attached and light turned on) against the palate of the mouth (with the curved slide facing downward) while holding and deflecting the tongue to the side. Next, place a drop of lidocaine gel to the tip of the lidocaine applicator and apply it the epiglottis (arch shaped and located just above the vocal cords) while still holding the otoscope and speculum against the palate of the animal's mouth with the opposite hand. Lidocaine gel application should reduce epiglottis spasm as well as irritation to this sensitive region. Put animal back on anesthesia mask (rats 2.5% Iso; AGS 3-3.5% Iso) for 2 min.	
5. **Take animal off mask and reinsert otoscope/speculum unit as described earlier.** This time use the endotrachael catheter (14ga 1.75in; BD Insyte Autoguard, shielded iv catheter, ref# 381467 guided with a bent, custom blunt biomedical 17gax4in needle, 17TWx3, Popper & Sons, New Hyden Park, NY #7427) and try to insert it (with curved side facing up) into the trachea located via the opening between the two vocal cords. Movement of the vocal cords should be slowed down enough in order to maneuver this technique. Once the catheter is fully inside the trachea (only the orange hub should be sticking out of mouth), quickly check if the catheter is truly in the trachea and not in the esophagus by holding a stainless steel instrument in front of the open end of the catheter (orange hub) so that condensation of respiratory moisture can be seen. If no moisture is visible, then it means that the catheter is in the esophagus. Repeat aforementioned steps in order to reinsert endotracheal catheter into trachea. Discontinue surgery if more than 2 tries are needed to execute intubation. Return animal to home cage for at	

Figure A-2. "Surgery Log" form page 2. Shows anesthesia induction and intubation preparation.

least 1 week before repeat attempt. If necessary, trachea may be visualized to facilitate intubation. Clip the hair from the ventral neck and using a #10 or #15 blade or curved, blunt scissors make a 1-2 cm incision along the ventral midline of the neck to expose the trachea and larynx. To prevent laryngospasm during oral intubation swab lidocaine onto laryngeal folds with a small cotton tip swab moistened with 2 drops of lidocaine (Lidoject; lidocaine 2% injectable, Butler Animal Health Supply, Dublin, OH 43107). Close with Prolene, 3-0 suture, simple interrupted stitch. Suture catheter to lip to avoid dislodging catheter during resuscitation. Close with 3-O prolene, simple interrupted stitch, at least 3 throws). For terminal, non-recovery procedures a tracheotomy may be substituted for oral intubation. The trachea will be exposed as described above. A small hole will be made between the cartilaginous rings in the trachea and the 14ga 1.75in; BD Insyte catheter will be inserted. The catheter will be secured in the trachea with1 or 2 sutures using 3-0 silk.	
6. **Shave** throat (if tracheotomy is planned), right groin, and *(optional)* dorsal surface of remaining legs (for ECG electrodes); scrub animal using 70% alcohol and 100% betadine solution beginning with betadine and alternating with 70% isopropyl alcohol for 3 times (three swabs betadine and two swabs 70% alcohol), ending with 100% betadine. Use circular, inside-to-outside motions when scrubbing incision so as not to contaminate the innermost region.	
7. **Record vitals:** Cover eyes with sterile ophthalmic ointment for lubrication (e.g. vetropolycin, neo-poly-bac, etc.), insert rectal thermometer (lubricated with K-Y), insert EKG leads subcutaneously (ground to hind leg; negative to left front leg, positive to right front leg) and secure both EKG leads and thermocouples/needles with surgical tape; monitor sO2 with pulse-oximeter if applicable. Record values on surgery log and anesthesia record.	
8. Don sterile gloves, drape and open pack[1] and arrange surgical instruments. **Note* If tracheotomy is planned: o IV catheter (same for rats and AGS; BD/Insyte; H3124A; 14ga x 1.75 inch; 2.1 x 45mm) o 3-0 Prolene (when closing)	

Figure A-3. "Surgery Log" form page 3. Intubation and surgical preparation setup shown.

9. Cannulate femoral vein and then artery, flush with saline and close temporarily with sutures. Polyethylene catheters (Tygon tubing; 0.375-mm ID, 0.75-mm OD; Norton, Akron, OH or PE-50 catheter for vein and artery), will be introduced ~ 2 cm into the right femoral artery and vein for acute blood pressure recording, blood sampling, and drug infusion; or 6cm for chronic cannula placement. Mean arterial blood pressure (MABP) will be measured via an indwelling femoral arterial catheter connected to a precalibrated Statham pressure transducer and will be recorded continuously. Arterial blood gases and pH, and plasma glucose, will be measured in microsamples (75-100μL). (Total volume not to exceed 1.5mL.	
10. Intubation (for AGS only endotrachael intubation must follow venous cannulation because vecuronium must be administered immediately after intubation or AGS breathe against the endotrachael catheter): *See **step 4** above*.	
11. Paralyze: AGS must be paralyzed *immediately* after inserting endotrachael catheter. Inject 0.3mL Vecuronium (1mg/mL) iv. Watch to see that animal stops breathing. Connect ventilator (humidified with mucomist (1.4mL/70mL H2O); pressure dampened with rebreathe bag at, approximately 53 rpm, SV 2.0mL). Immediately decrease isoflurane to 0.5%.	
12. Sample and adjust blood gases; adjust stroke volume, SV, (typically between 2 – 3 mL) and %O2 (typically between 450 and 500mL/min) to adjust blood gases (AGS PCO2: 30-84mmHg; PO2 41-73 mmHg[PCO2 of 60mmHg and PO2 of 60mmHg is typical for AGS]; Rat PCO2: 35-40; PO2 100-110 [110-130 is acceptable][1]	
13. Monitor T_{rec} and $T_{temporalis}$ until between 36.5 and 37.5°C.	
14. At least 10 min after previous injection of vecuronium, inject vec (0.3mL of 1mg/mL, iv) and FLUSH immediately with 0.3mL hep-sal (6.7 IU/mL), 2 min later disconnect ventilator. OBSERVE THAT ANIMAL IS NOT BREATING. If animal continues to breathe (>2 breaths) inject another 0.3mL of vec, wait 10min, and repeat this step. If animal is not breathing prepare to resuscitate[2]. (Connect 2 way luer adapter primed with Epi (5ug/mL) and NaHCO3 (8.4%) for immediately delivery; change BP scale to 0-50; turn off isoflurane vaporizer; turn N2O flow meter to 0 and increase O2 to 2L/min and respiratory rate to 80 bpm. NOTE last vec injection and start of CA or sham procedure and Resuscitation in LabScribe.	
15. Resuscitate by reconnecting ventilator, immediately inject 1mL/kg fresh Epi (5ug/kg or 10ug/kg). Immediately inject Epi and apply rapid manual chest compressions at a rate of 200/min to base of the heart until BP is at least 50 mm Hg and is	

Figure A-4. "Surgery Log" form page 4. Cannulation, blood gas sampling, cardiac arrest induction, and resuscitation step-by-step procedures shown.

maintained by spontaneously beating heart for > 10 sec. If not resuscitated after 1 min, give a second Epi dose. Once HR is spontaneous and MABP > 50 mm Hg, immediately inject 0.9 cc NaHCO3. Resuscitation is discontinued after 2 min if animal fails to maintain a spontaneously beating heart. Squishing sounds from lung during manual compression is indication of lung injury and may be used as a basis for euthanasia.	
16. Immediately sample blood gases once animal maintains stable MABP after restoration of spontaneous circulation (ROSC). After 10 minutes of ROSC, decrease respiratory rate from 80 to approx. 60 bpm and %O2 from 100% to 70% in a mixture with N2O. Again sample arterial blood gases.	
17. Correct acid-base status with sodium bicarbonate and/or the ventilator settings. (Base excess should be >0; 5-10 is normal). If pH<7.3 inject NaHCO3 to adjust pH. May need to increase RR to blow off CO2 if pH is low, in AGS CO2 is often >60. Stroke volume must be >2mL.	
18. Once blood gases are normal, remove arterial and venous catheters by tying off artery and vein with 3 throws of 3-0 silk suture. Close skin with 3-O prolene, simple interrupted stitch; at least 3 throws. **Inject Sterile Saline (i.p. 1mL/kg)**. Or, if **chronic cannula** is needed go to chronic arterial/venous cannulation SSOP.	
19. Insert IPTT tag, if not present.	
20. Leave on ventilator until spontaneous breathing begins to fight ventilator.	
21. Maintain at 37°C (between 36.5 and 37.5°C) for at least 60 min. Animals not able to maintain 36-37.5°C body temperature without heat lamps are placed in a humidified neonatal incubator set at 29°C for 12-24h or until animals regain normal thermoregulation.	
22. Place animal in postoperative cage (with underpad and water bottle provided) and put in neonatal incubator set to 29°C once animal spits out endotracheal catheter own their own (or with some help by the surgeon if catheter is still lodged in throat due to mucus secretions). Do not provide any food in the cage as this can sometimes lead to accidental death.	
23. Copy and file anesthesia records, surgery logs, data sheets, animal care log sheets, and neurological deficit scores (NDS) sheets into appropriate binders and locations. Enter blood gas data into Excel, backup LabScribe files to both laptop and designated external hard drive.	
24. Monitor a minimum of 3 times daily per post-op care below until animal shows signs of voluntary feeding, cleaning, and is able to easily move around in cage on their own.	
25. Record body temp daily and inspect/clean sutures daily up to 7 days and at regular intervals thereafter. Remove sutures at 10-12 days.	

Figure A-5. "Surgery Log" form page 5. Post-ROSC (resuscitation) steps outlined for cardiac arrest surgery.

POST-OPERATIVE CARE: (describe care and monitoring procedures, you must note the times that the animal regains righting reflex and becomes ambulatory)

Post-op care: Animal will be maintained in a neonatal incubator set to 29.0°C for 24hr after surgery. Animal will be placed in a clean, stainless steel cage with an underpad provided and water bottle, but **NO** wood shavings. **If animal is ambulatory and unaffected by surgery, animal will be fed fresh carrots and apples, rodent chow, and water and returned to its home room.**

Personnel approved for post-operative care and monitoring will participate in:

Name(s):_____

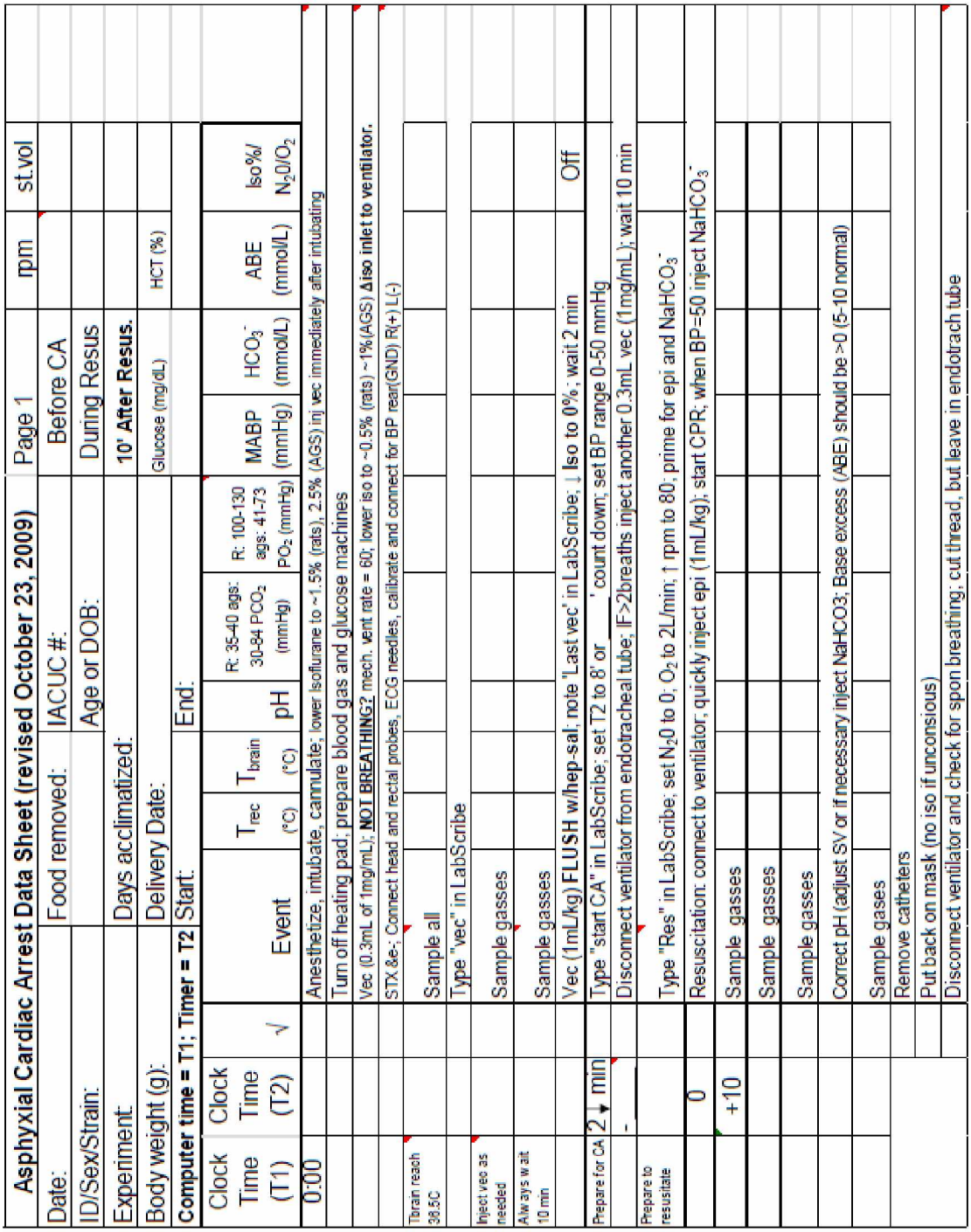
Post-Op Care	Note Date, Time & Observations
Daily cleaning and inspection of wounds. Wounds are cleaned with dilute (tea colored) betadine once per day for 3 days and inspected thereafter.	
8- 16 h post-op, animals are observed for signs of distress and fed rodent chow soup mixture placed in a petri dish. If not able to eat at all on their own, animals are **fed by gavage (1ml/kg body weight) 3 times per day:** (The stomach volume is about 3 ml in about 300 grat and gastric emptying time about 45 - 60 min. Sucrose (i.e., table sugar from grocery store) is mixed ~50:50 with chow and dissolved with tap water to make a dilute solution that can be drawn into a 3 cc syringe using a gavage needle without clogging the needle). Animals that respond and swallow are fed by placing the tip of the gavage needle into the mouth and allowed to consume more than 1mL/kg. Animals not consuming at least 3mL/kg are fed by gavage.	
Beginning at 8-16 h post-op **body temperature** is recorded and animals are monitored for neurological impairment and scored per "**Neurological Deficit Scores (NDS)" daily for the first 7 days** and at regular intervals thereafter.	
Vet services will be contacted in case of complications encountered during regular work hours or during after-hour, weekend or holiday care (e.g. wound infection or unusual behavior by animal).	
Although the severity of brain damage is not expected to produce death, inter-animal variation makes death a possibility. **Animals will be monitored, fed by gavage (1mL/100g body weight) and given supportive care as needed at least 3 times per day**. Severity of brain damage may be assessed from neurological examination. Animals showing severe neurological impairment including coma will not be euthanized because eliminating most severely affected individuals will bias final assessment of histology. The number of animals that die will be noted, however, these animals are excluded from histological examination of tissue because death does not allow for in situ fixation of the tissue. Deposit dead animals in refrigerator in AQ office, leave a note that the carcass is in the refrigerator, and as always, update record on AQ disposition log and in lab animal inventory notebook.	

***NOTE: Use back of page if necessary. Any mortality or complication must be submitted to Veterinary necropsy.**

Figure A-6. "Surgery Log" form page 6. Post-operative care procedure outlined for rats recovering from cardiac arrest surgeries 8-16hr post-resuscitation.

Appendix B

Asphyxial Cardiac Arrest Data Sheet



Asphyxial Cardiac Arrest Data Sheet (revised October 23, 2009)				Page 1		rpm	st.vol
Date:	Food removed:	IACUC #:		Before CA			
ID/Sex/Strain:		Age or DOB:		During Resus			
Experiment:		Days acclimatized:		**10' After Resus.**			
Body weight (g):		Delivery Date:		Glucose (mg/dL)		HCT (%)	
Computer time = T1; Timer = T2		Start:		End:			

Clock Time (T1)	Clock Time (T2)	√	Event	T_{rec} (°C)	T_{brain} (°C)	pH	R: 35-40 ags: 30-94 PCO_2 (mmHg)	R: 100-130 ags: 41-73 PO_2 (mmHg)	MABP (mmHg)	HCO_3^- (mmol/L)	ABE (mmol/L)	Iso%/ N_2O/O_2
0:00			Anesthetize, intubate, cannulate; lower isoflurane to ~1.5% (rats), 2.5% (AGS) inj vec immediately after intubating									
			Turn off heating pad; prepare blood gas and glucose machines									
			Vec (0.3mL of 1mg/mL); **NOT BREATHING?** mech. vent rate = 60; lower iso to ~0.5% (rats) ~1%(AGS) **Δiso inlet to ventilator.**									
			STX &e-; Connect head and rectal probes, ECG needles, calibrate and connect for BP rear(GND) R(+) L(-)									
Tbrain reach 38.5C			Sample all									
			Type "vec" in LabScribe									
Inject vec as needed			Sample gasses									
Always wait 10 min			Sample gasses									
			Vec (1mL/kg) **FLUSH w/hep-sal**; note 'Last vec' in LabScribe; ↓ Iso to 0%; wait 2 min									Off
Prepare for CA	2 ↓ min		Type "start CA" in LabScribe; set T2 to 8' or ___' count down; set BP range 0-50 mmHg									
	-		Disconnect ventilator from endotracheal tube; IF>2breaths inject another 0.3mL vec (1mg/mL); wait 10 min									
Prepare to resusitate			Type "Res" in LabScribe; set N_2O to 0; O_2 to 2L/min; ↑ rpm to 80; prime for epi and $NaHCO_3^-$									
	0		Resuscitation: connect to ventilator, quickly inject epi (1mL/kg); start CPR; when BP=50 inject $NaHCO_3^-$									
	+10		Sample gasses									
			Sample gasses									
			Sample gasses									
			Correct pH (adjust SV or if necessary inject NaHCO3; Base excess (ABE) should be >0 (5-10 normal)									
			Sample gases									
			Remove catheters									
			Put back on mask (no iso if unconsious)									
			Disconnect ventilator and check for spon breathing; cut thread, but leave in endotrach tube									

Figure B-1. "Asphyxial Cardiac Arrest Data Sheet" form page 1. This form is used to record physiological values obtained from rats during cardiac arrest surgery.

Asphyxial Cardiac Arrest Data Sheet (revised October 23, 2009) Page 2

Date:			**Animal ID #:**		
			Maintain at T_b at 37.0°C for 60 min		
Checklist		√	Task		NOTES
			Give i.p. 1mL/kg Sterile Saline (non-heparanized)		
			Put in humidified incubator at 29.0°C overnight		
Clock time	Date		Next morning: feed 3mL sugar water chow mix 3 times /day		
			am feeding		
			noon feeding		
			evening feeding		
			Record T_{rec} Daily	°C	Body wt (g)
			24-hr post resus		
			48-hr post resus		
			72-hr post resus		
			Perfuse for histology		
			1'Saline →19' FAM & Head in FAM for 24hr; Head rinsed w/RO H_2O & Brain in FAM 24hr; Brain in 70% EtOH until ready for trim		
			Dissect out brain and put into FAM at 4°C at least overnight; store at 4°C in FAM until parafin embedding		
			Embedded		

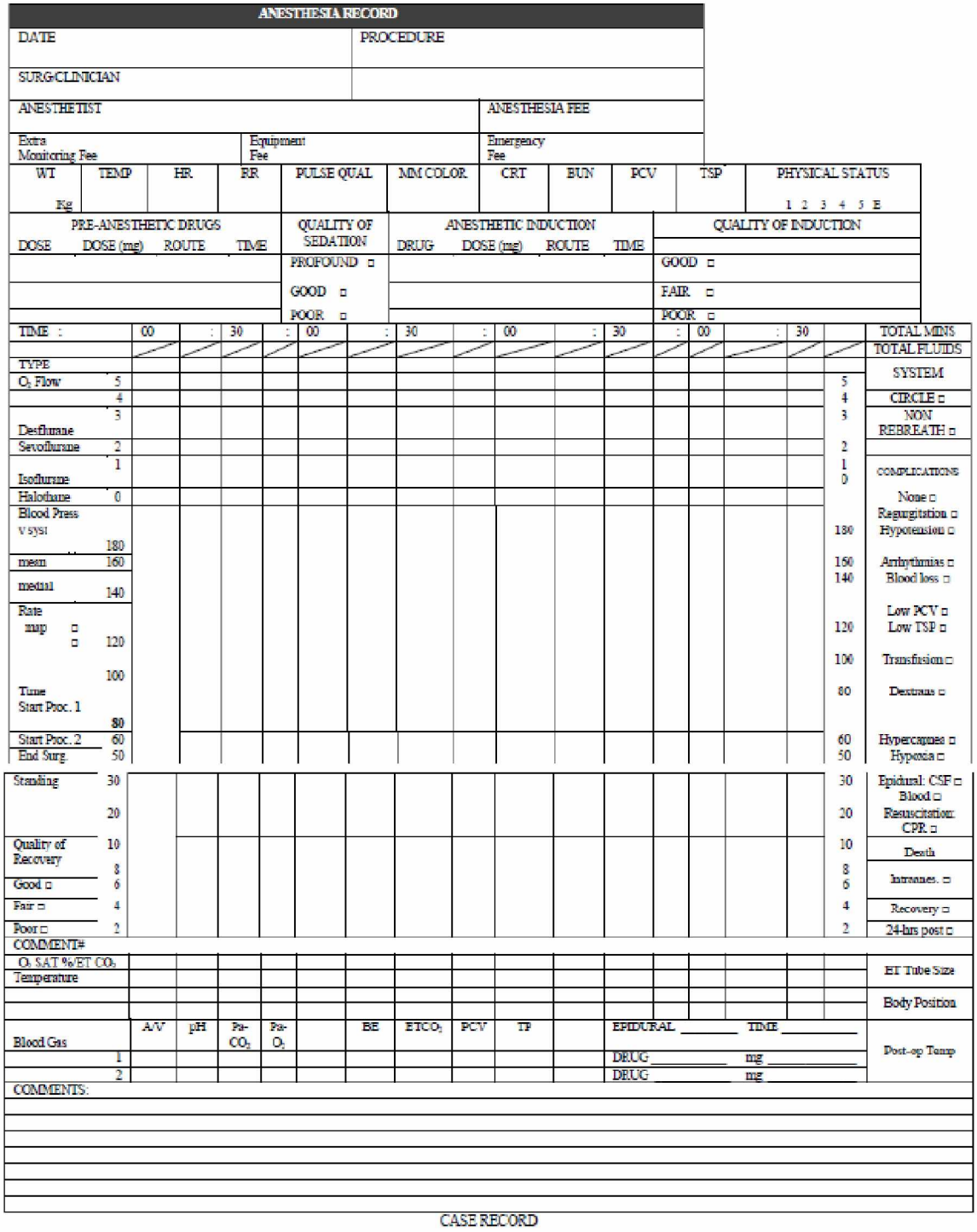
Additional Time Points

Clock Time (T1)	Clock Time (T2)	√	Event	T_{rec} (°C)	T_{brain} (°C)	pH	R: 35-40 ags: 30-84 PCO_2 (mmHg)	R: 100-130 ags: 41-73 PO_2 (mmHg)	MABP (mmHg)	HCO_3^- (mmol/L)	ABE (mmol/L)	Iso%/ N_20/O_2

Figure B-2. "Asphyxial Cardiac Arrest Data Sheet" form page 2.

Appendix C

Anesthesia Log



ANESTHESIA RECORD

DATE	PROCEDURE
SURG-CLINICIAN	
ANESTHETIST	ANESTHESIA FEE
Extra Monitoring Fee	Equipment Fee / Emergency Fee

WT Kg	TEMP	HR	RR	PULSE QUAL	MM COLOR	CRT	BUN	PCV	TSP	PHYSICAL STATUS 1 2 3 4 5 E

PRE-ANESTHETIC DRUGS DOSE	DOSE (mg)	ROUTE	TIME	QUALITY OF SEDATION	ANESTHETIC INDUCTION DRUG	DOSE (mg)	ROUTE	TIME	QUALITY OF INDUCTION
				PROFOUND ◻					GOOD ◻
				GOOD ◻					FAIR ◻
				POOR ◻					POOR ◻

TIME :	00	:	30	:	00	:	30	:	00	:	30	:	00	:	30		TOTAL MINS
																	TOTAL FLUIDS
TYPE																	SYSTEM
O_2 Flow 5																5	
4																4	CIRCLE ◻
Desflurane 3																3	NON REBREATH ◻
Sevoflurane 2																2	
Isoflurane 1																1	
Halothane 0																0	COMPLICATIONS
Blood Press v syst																	None ◻ Regurgitation ◻
180																180	Hypotension ◻
mean 160																160	Arrhythmias ◻
mean1 140																140	Blood loss ◻
Rate map ◻ ◻ 120																120	Low PCV ◻ Low TSP ◻
100																100	Transfusion ◻
Time Start Proc. 1																80	Dextrans ◻
80																	
Start Proc. 2 60																60	Hypercapnea ◻
End Surg. 50																50	Hypoxia ◻
Standing 30																30	Epidural: CSF ◻ Blood ◻
20																20	Resuscitation: CPR ◻
Quality of Recovery 10																10	Death
8																8	
Good ◻ 6																6	Intraanes. ◻
Fair ◻ 4																4	Recovery ◻
Poor ◻ 2																2	24-hrs post ◻
COMMENT#																	
O_2 SAT %/ET CO_2																	ET Tube Size
Temperature																	
																	Body Position
Blood Gas	A/V	pH	Pa-CO_2	Pa-O_2		BE	$ETCO_2$	PCV	TP		EPIDURAL _____ TIME _____						
1											DRUG _____ mg _____						Post-op Temp
2											DRUG _____ mg _____						
COMMENTS:																	

CASE RECORD

Figure C-1. "Anesthesia Log" form shown. This form records physiological parameters of anesthetized rats during cardiac arrest surgery.

Appendix D

Asphyxial Cardiac Arrest Setup

Asphyxial Cardiac Arrest Setup
March 13, 2007

Order

- (±) Epinephrine HCl (Epinephrine injection, 1mg/mL, Butler Columbus Ohio, cat #WAB10310) (store at 4°C)
  - Dilute 1:50 to make 0.05 mg/mL: To red top tube add 0.5 mL of 1mg/mL epi + 9.5 mL saline to make 0.05 mg/mL (50 ug/mL)
  - Dilute 1:10 to make 0.005 mg/mL: To red top tube add 1.0 mL of 0.05 mg/mL + 9.0 mL sterile saline.
- CG8+ iSTAT Cartridges (3 mon shelf life) http://www.abbottpointofcare.com/istat/www/products/cartridges.htm, Abbott Laboratories. Abbott Park, Illinois
- Contact Abbott labs to order blood gas standards (Calibration solutions are contained within each cartridge, but it is best to confirm accuracy with known standard especially if cartridges are near or exceed shelf life).

Gas sterilize (24hr prior to surgery; aerate overnight) for survival or acute surgery. Assemble pressure transducer using sterile technique, fill with sterile saline and use for up to 1 week or until contaminated. Replace line to arterial cannula daily with gas sterilized line.

Or Cold sterilize for acute surgery:

- 2, x~20 cm PE-50 or Tygon tubing (0.375-mm ID, 0.75-mm OD; Norton, Akron, OH; special order) cannulae (marked 1 inch at one end and 2-3inches from other end) with blunt stainless steel fittings (23ga for PE-50; 26ga for Tygon tubing);
- Pressure gauge and PE-90 x 50-70 cm tube with connectors to connect 10cc syringe and to arterial line for BP monitoring

Autoclave: Double wrapped pack[1] (see below)

Fast rats and AGS 20-24hr before surgery by moving animal to clean, food free cage 20-24hr prior to procedure

Set out:

- Injectables (see below) with color coded syringes for 2x-HEP sal, Vec, $NaHCO_3$ and Epi

- ☐ 10mL syringe filled with sterile saline and bottle of sterile saline
- ☐ Sterile 10 cc syringe (to fill with hep-sal and connect to pressure gauge)
- ☐ 2 x 5 inch curved hemostats with Tygon on tips
- ☐ Water blanket covered with towel or absorbent blue pad-Warm up
- ☐ Clippers
- ☐ Balance for weighing animal
- ☐ Curved forceps for grapping tongue
- ☐ Filled isoflurane vaporizer; Flow meter for N_2O and O_2; tanks of N_2O and medical grade O_2 with jar or box for inducing anesthesia
- ☐ 2 lamps with 60-75 W bulbs; each connected to Omega (CSC32 temp controllers; calibrated with thermocouples to be used for experiment with Omega HH41 digital thermometer and ON-403-PP thermocouple)
- ☐ Needle thermocouple
- ☐ Rectal thermocouple and lubricant
- ☐ ECG needles (Platinum; F-EZ; 12 inch; 10/box)
- ☐ DSI transducers (bioelectric for ECG set to 0.1; transducer for BP; output (0-1V; set to 100) connected to gas sterilized pressure gauge; 10 cc syringe filled with hep-sal and 50-70 cm inlet (of same tubing as cannula) with blunt luer connector to connect to BP transducer and blunt stainless steel adapter to connect to arterial line for BP monitoring
- ☐ Ring stand for holding pressure gauge
- ☐ Pressure meter (such as physicians use for BP monitoring)
- ☐ Computer with I-works A-D converter and software
- ☐ Ventilator [*Recommended ventilator*: ***UGO Basile Rodent Ventilator***, *Stoelting Cat# 50095, $4850.* ***Harvard ventilator*** *does not provide reliable volume.* CO_2 *may gradually increase and be difficult to regulate*] (set to appropriate stroke volume and rpm with 0.5L bag (Csharkey@vital-signs.com) in loop to serve as pulse dampener for lungs); connected to 250 mL humidifier (Scienceware, www.belart.com, cat#110300000) filled with 25mL H_2O + 20uL mucomist (acetylcysteine 10%; Roxane laboratories, Cincinnati, OH; NDC 0054-3027-02). Cylinder is capped with 2-holed rubber stopper. Hard Teflon, ~1/4in ID inlet tube with 90^o bend reaches into fluid at bottom. Similar, but shorter outlet tube feeds humidified air into ~1/4 in ID Tygon. Outlet connects to inlet on ventilator. Ventilator outlet connects to ~1/8 in ID T that connects to endotracheal luer lock hub and ventilator inlet to complete circuit of humidified air. O_2 and N_2O tanks to flow meter to isoflurane vaporizer to humidifier to fluovac inlet (clamp 2^{nd} inlet for 2^{nd} line) to animal. When animal is on ventilator inlet from humidifier goes to inlet on ventilator. Outlet from ventilator goes to Omnicon f/air canister or for UGO Basil ventilator fluovac mask fits in hole for ventilator output and suction scavenges output.
- ☐ Fluovac or other scavenger and mask
- ☐ 2-3 sets of sterile surgical gloves
- ☐ Betadine (full strength) and 70% isopropyl alcohol for scrubbing

- ☐ Gauze for scrubbing
- ☐ Q-tips for scrubbing
- ☐ Data sheet, post-op recovery sheet or tissue collection sheet; and, UAF (surgery log; anesthesia record); UM (protocol sheet and animal ID number recorded in lab log)
- ☐ Surgical microscope or magnifying glasses
- ☐ Blood gas machine and blood glucose machine or iSTAT machine with cartridges for blood gases and glucose and hematocrit centrifuge
- ☐ UM (500uL vials for glucose; clay capillary tubes for HCT; long capillary tube and 2 caps for blood gases); UAF (capillary tubes and putty for HCT; same capillary tubes for blood gases and glucose via iSTAT), AND 500uL heparinized microfuge tubes to collect 500uL blood, spin and freeze plasma for hormone analysis.

Procedure:

1. **Open pressure gauge pack and set up pressure transducer.**
2. **Prepare injectables** (see list near end of this document) **and fill two cannulae w/hep-saline** w/1cc syringe and 23ga. luer stub adapter (Becton Dickinson; reorder # 427565) for PE-50; 26ga luer stub for Tygon. Fill one 10cc syringe w/sterile saline. Leave sterile ends in pack.

3. **Anesthetize** with 5% isoflurane and a 30:70 mixture of O_2 (400-500mL/min) and N_2O (1L/min) to insure physiological levels for PO_2, PCO_2 and pH.

4. **Shave** throat, right groin, and dorsal surface of remaining legs (for ECG electrodes); scrub animal.

5. **Record vitals:** Cover eyes with lubricant, insert rectal thermometer (lubricated with K-Y), insert EKG leads subcutaneously (ground to hind leg; negative to left front leg, positive to right front leg), monitor sO2 with pulse-oximeter. Record values on anesthesia record.

6. **Don sterile gloves, drape and open pack[1] and arrange surgical instruments.**
   [1](double wrap is sterile for 6 weeks; single wrap is sterile for 3 weeks)

- ☐ Small vessel scissors (protect with tip of yellow pipette)
- ☐ 45^o 5/45 sharp forceps (protect with tip of yellow pipette)
- ☐ Blunt; bent 16ga/2.5" needle (may bend, blunt and file needle from IV catheter used for endotracheal catheter) or purchase from Popper & Sons, 17TWx3 custom blunt popper biomedical needle (cat# 7427, http://popperandsons.com/index.asp)
- ☐ Fine, straight rat-toothed forceps (Miltex 6-106; ~1mm or 1/16" width x 5 inches long)
- ☐ 90^o curved hemostats (5.5 inch long; Roboz RS 7291)

- 2 x 8 inch smooth inner surface curved (~90°) curved forceps (for teasing apart and holding vessels) Paragon #4004-96 http://www.paragonmedical.com/
- Curved, blunt scissors for cutting skin and separating skin and muscle (Roboz; RS-6891)
- 4 inch curved forceps for holding cannula (smooth, I used rough at UM, but tear up surface of cannula) and 2 ½“ forceps (rough)
- Needle holder
- Clear plastic drape with hole for throat and loin
- Y-Luer adapter
- Whole pack of cotton tipped applicators wrapped in tin foil
- Retractor for throat
- Small scissors for cutting knots
- ½” pile of gauze wrapped in tin foil (Flush with saline, clean out blood clots, cover with saline wetted gauze)
- Extra, 4 x 23ga blunt connectors, 6 x 23ga. Luer stub adapters (for PE-50) 6x 26ga luer stub adapters (for Tygon tubing)
- 1 razor blade per pack
- 1 silk needle w/suture
- 4 x 15 inch pieces of silk thread
- Glass slide covered w/foil
- Follow procedure on Excel data sheet

7. Open into sterile field

- IV catheter (same for rats and AGS; BD/Insyte; H3124A; 14ga x 1.75 inch; 2.1 x 45mm)
- 3-0 Prolene (when closing)

8. Cannulate femoral vein and then artery, flush with saline and close temporarily with sutures. Polyethylene catheters (Tygon tubing; 0.375-mm ID, 0.75-mm OD; Norton, Akron, OH or PE-50 catheter for vein and artery), will be introduced ~ 2 cm into the right femoral artery and vein for blood pressure recording, blood sampling, and drug infusion. Mean arterial blood pressure (MABP) will be measured via an indwelling femoral arterial catheter connected to a pre-calibrated Statham pressure transducer and will be recorded continuously. Arterial blood gases and pH, and plasma glucose, will be measured in microsamples (75μL).

9. Intubate with endotrachael catheter (14ga 1.75in; BD Insyte Autoguard, Shielded IV Catheter, ref# 381467 guided with a bent, custom made blunt biomedical 17ga x 4in needle, 17TWx3, Popper & Sons, New Hyden Park, NY #7427). If necessary, clip the hair from the ventral neck and using a #10 or #15 blade or curved, blunt scissors make a 1-2cm incision along the ventral midline of the neck to expose the trachea and larynx. To prevent laryngospasm during oral intubation swab lidocaine onto laryngeal folds with a small cotton tip swab moistened with 2 drops of lidocaine

(Lidoject; lidocaine 2% injectable, Butler Animal Health Supply, Dublin, OH 43107). For survival surgery, suture the catheter to the lip to avoid dislodging catheter during resuscitation CPR. Close with 3-O prolene, simple interrupted stitch, at least 3 throws).

For terminal, non-recovery procedures, a tracheotomy may be substituted for oral intubation. The trachea will be exposed as described above. A small hole will be made between the cartilaginous rings in the trachea and the 14ga 1.75in; BD Insyte catheter will be inserted. The catheter will be secured in the trachea with1 or 2 sutures using 3-0 silk.

10. **Paralyze** AGS must be paralyzed *immediately* after inserting endotrachael catheter. Inject 0.3cc Vecuronium (1mg/mL) IV. Watch to see that animal stops breathing. Connect ventilator (humidified with mucomist (1.4mL/70mL H_2O); pressure dampened with rebreath bag at, 60 rpm, SV 2.5mL). Decrease isoflurane to 0.5%.

11. **Sample and adjust blood gases;** adjust stroke volume, SV, (typically between 2 – 3 mL) and $\%O_2$ (typically between 250 and 600mL/min) to adjust blood gases (AGS PCO_2: 30-84mmHg; PO_2 41-73mmHg [PCO_2 of 60mmHg and PO_2 of 60mmHg is typical]; Rat PCO_2: 35-40; PO_2 100-110 [110-130 is acceptable][1].

12. **Monitor T_{rec} and $T_{temporalis}$** until between 36.5°C and 37.5°C.

13. **At least 10 min after previous injection of vecuronium**, inject vec (0.3mL of 1mg/mL, IV) and FLUSH immediately with 0.3mL hep-sal (6.7 IU/mL), 2 min later disconnect ventilator. OBSERVE THAT ANIMAL IS NOT BREATHING. If animal continues to breathe (>2 breaths), inject another 0.3mL of vec, wait 10min, and repeat this step. If animal is not breathing prepare to resuscitate[2]. (Connect 2 way luer adapter primed with Epi (5ug/mL) and $NaHCO_3$ (8.4%) for immediately delivery; change BP scale to 0-50; turn off isoflurane vaporizer; turn N_2O flow meter to "0" and increase O_2 to 2L/min and respiratory rate to 80 bpm. NOTE "last vec" injection and "start of CA" or "sham" procedure and "res" (resuscitation) in LabScribe. Setup "PEEP" (Positive End-Expiratory Pressure) if applicable—place the expiratory end of ventilator tubing against the bottom of a container filled with 5 cm of H_2O).

14. **Resuscitate** by connecting ventilator, inject 1mL/kg fresh Epi (5ug/kg). Pull back on $NaHCO_3$ plunger when injecting Epi; apply rapid heart message until BP is at least 50 and HR is spontaneous (for at least 10 sec). Immediately inject 0.9 cc $NaHCO_3$. Resuscitation is discontinued after 2 min if animal fails to maintain a spontaneously beating heart.

15. After 10 minutes of restoration of spontaneous circulation (ROSC), decrease respiratory rate from 80 to 60 bpm and $\%O_2$ from 100% to 70% in a mixture with N_2O. Sample arterial blood gases.
16. Correct acid-base status with sodium bicarbonate and/or the ventilator settings. (Base excess should be >0; 5-10 is normal). If pH<7.3 inject $NaHCO_3$ to adjust pH. May need to increase RR to blow off CO_2 if pH is low, in AGS CO2 is often >60.
17. Once blood gases are normal, **remove arterial and venous catheters** by tying off artery and vein with 3 throws of 3-0 silk suture. Close skin with 3-O prolene, simple interrupted stitch; at least 3 throws.
18. Leave on ventilator until spontaneous breathing begins to fight ventilator.
19. Maintain at 37.0°C (between 36.5°C and 37.5°C) for 60 min.
20. Monitor until ambulatory.
21. Return to home cage.
22. Record body temp and inspect sutures every 24hr for 3 days.
23. File anesthesia records, surgery logs and data sheets.
24. Enter blood gas data into Excel.
25. Back up LabScribe files on laptop and to an external hard drive.

Post-op Care:
Personnel approved for post-operative care and monitoring will participate in the following:

1. Daily cleaning and inspection of wounds. Wounds are cleaned with dilute (tea colored) betadine once per day for 3 days and inspected thereafter.
2. 8-16hr post-op, animals are observed for signs of voluntary feeding. If not eating, animals are fed by gavage (1mL/kg body weight) 3 times per day: (The stomach volume is about 3mL in about 300g rat and gastric emptying time about 45-60min. Sucrose (i.e., table sugar from grocery store) is mixed ~50:50 with chow and dissolved with tap water to make a dilute solution that can be drawn into a 3 cc syringe using a gavage needle without clogging the needle).
3. 8-16hr post-op and again at 24hr intervals for 7 days, body temperature is recorded and animals are monitored for neurological impairment and scored per "Neurological scores data sheet.doc".
4. Vet services will be contacted in case of complications encountered during regular work hours or during after-hour, weekend or holiday care.
5. Although the severity of brain damage is not expected to produce death, inter-animal variation makes death a possibility. Animals will be monitored, fed by gavage (1mL/100g body weight) and given supportive care as needed up to 3 times per day. Severity of brain damage may be assessed from neurological examination. Animals showing severe neurological impairment including coma will not be euthanized because eliminating most severely affected individuals will bias final assessment of histology. Death must therefore be used as an endpoint. The number of animals that die will be noted, however, these animals are excluded from histological examination of tissue because death does not allow for in situ fixation of the tissue. Deposit dead animals in refrigerator in the Animal

Quarters (AQ) office, leave a note that the carcass is in the refrigerator, and as always, update record on AQ disposition log and in lab animal inventory notebook.

Notes:

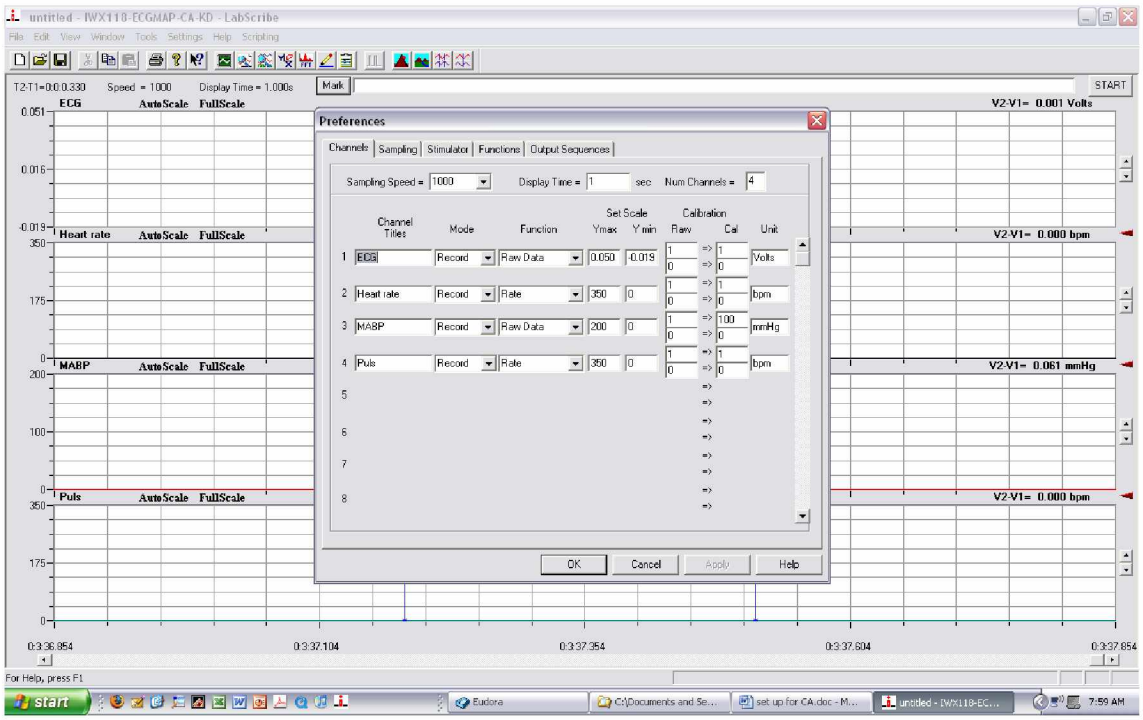
[1] Ventilatory volume will typically be 2.5mL and ventilatory rate will be 60 bpm prior to CA and 80 bpm after resuscitation. However rate and volume may vary as necessary to adjust blood gases and pH. Volume and rate will generally vary between 2.5mL ±1.0 mL and 60 ±10bpm (prior to CA); 80 ±10 after resuscitation. However both rate and volume may exceed these parameters if necessary to adjust blood gases and pH.

[2] An immediate bradycardia is typically observed followed by hypotension to 50mmHg. Within approximately three minutes, MAP decreases to zero mmHg and the heart rate is further decreased. Between 6 and 8 minutes of asphyxia, there is minimal ECG activity. Eight minutes (or longer depending on experimental protocol) after asphyxia, resuscitation will be initiated. After 10 minutes of restoration of spontaneous circulation (ROSC), the ventilator rate will be decreased to 60 bpm and the oxygen will be lowered to 70% in a mixture with N_2O. Arterial blood gases will then be determined and if any corrections in acid-base status are necessary, sodium bicarbonate will be administered, and/or the ventilator settings will be adjusted.

LabScribe (Software) Data Acquisition:

Start LabScribe

1. **Load program:**
   Settings/load IWX118-ECGMAP-CA-KD
2. **Select program:**
   Settings/Select IWX118-ECGMAP-CA-KD
3. **Place electrodes (subcutaneously, SC); GND to back leg; (+) to R front leg; (-) to left front leg.**
4. **Set Bioelectric transducer to 2.5 (full scale), In LabScribe click on Start and Full Scale in channel 1 (ECG) to acquire. If noisy change mV to V**
5. **Calibrate pressure transducer once per day:** Fill with sterile Hep-Sal (0.2mL of 1000IU/mL/30mL vial saline or 6.7 IU/mL) and bleed air from system. IN LabScribe/ Edit/preferences Values printed below should be seen in IWX118-ECGMAP-CA-KD



Open transducer to atmosphere; Press Shunt CAL, Press Auto Bal. LabScribe screen should read ~0 (+/- 1mmHg). If auto bal does not stabilize at 0, air is in line. Flush air. May need to adjust CALIB knob if first time combining pressure transducer with Gould transducer box. Connect stethoscope pressure gauge to outlet on pressure transducer and increase pressure to 50, 100, 150; adjust CALIB knob on transducer to correct reading on LabScribe.

6. **Monitor BP:** Connect to arterial catheter; Clamp catheter with hemostats protected with Silastic tubing when BP is not being monitored; Close transducer to air, close to syringe, open to artery. Check readout on LabScribe. Must be connected to transducer with luer LOCK. Arterial pressure will cause blood to bleed into line and transducer if any leak in system.

<u>Omega Temperature Controller</u>:(Refer to Omega CN9300 Temp Controller users guide.pdf at http://www.omega.com/Manuals/manualpdf/M2897.pdf)
For model CSC32 (T type)
Omega.com
Program settings

- **Retain or change to factory settings (level 3; rSEt)**
- **hold ∧ and ∨ keys together for 3 sec to enter program mode**
- **Scroll through program options with ∧ or ∨ key until LEVEL appears**
- **Scroll through level # with ∧ or ∨ key**

1. Level 1: Set *Autotune* <u>(tune) to OFF</u>

2. Level 1: Set *Integral* time (Int.t) to OFF
3. Level 1: Set *SP1 Prop band*(*gain*)/*hyst* (bAnd) to 0.1
4. Level 1: Set *Cycle time or on/off* (CYC.t) to ON.OFF
5. Level 2: Set *Select display units* (unit) to ^{o}C
6. Level 2: Set *Select input sensor* (I nPt) to tc t
7. Level 2: Set *Display resolution* (diSP) to 0.1 degree
8. To lock: Level 3: scroll to (uEr) and press ∧ and ∨ keys together for 10 sec until display switches to LoC4. Press * and scroll through ∧ to select ALL

If you get lost in program mode, press ∧ and ∨ keys together for 3 sec to return to display mode. Check the INSTRUMENTS ADJUSTMENTS above and try again.

When in program mode, after 60 sec of key inactivity the display will revert to either (I nPt) none or, if the initial configuration has been completed, the measured value. Any settings already complete will be retained.

Calibrate at start of each new set of experiments and every time a different thermocouple is attached to a temperature controller. Calibrate at 3 points or more (such as 25, 37, 42) using +/- 0.1^{o}C Hg thermometer or Omega digital calibration thermometer.

Hg (^{o}C)	Controller 1 and Rectal probe (^{o}C)	Controller 2 and needle thermocouple (^{o}C)	Difference-1	Difference-2	% Error-1	% Error-2
41.5	39.7	39.6	1.8	1.9	4.337349	4.578313
39.5	37.7	37.9	1.8	1.6	4.556962	4.050633
32.2	30.6	30.7	1.6	1.5	4.968944	4.658385
27.3	25.7	25.8	1.6	1.5	5.860806	5.494505
25.9	24.5	24.5	1.4	1.4	5.405405	5.405405
25.2	23.8	23.9	1.4	1.3	5.555556	5.15873
		AVE	**1.6**	**1.5**	**5.11417**	**4.890995**

1. UNLOCK: Level 3: scroll to (uEr) and press ∧ and ∨ keys together for 10 sec until display switches to LoC4. Press * and scroll through ∧ to select nonE
2. Level 3: Set (*Zero) to difference (+ if controller is low; - if controller is high)*
3. Lock as in 8 above
4. Check that lamps turn off at 37.5^{o}C and on at 37.0 or higher. Note one output plug is for heating and the other is for cooling. If lamp does not turn off, move plug to other output.

Mechanical Ventilators:
R. Busto says Harvard is not a good model. Best is the UGO Basile (Italian company has booth at SFN) model. UGO Basile is more reliable and able to make fine adjustments. If Harvard ventilator goes bad we will be unable to control CO_2. CO_2 will tend to increase because of error in expiratory volume.

BP and EKG
EKG bioelectric lead: plug electrodes into **top** pin in house shaped plug 

Bioelectric transducer/ECG Lead Instructions:
1.) Try to make electrodes and wire harness as still and immobile as possible.

2.) Insert electrodes into animal using surgical tape if necessary.

3.) Turn on ECG box to "full scale" setting #1.

4.) Turn low cut filter to 10 and high cut filter to 30.

5.) Start LabScribe and click on the "autoscale" button.

6.) If ECG box is making a "clicking" sound, it is trying to figure stuff out. Give it some time to stabilize and try to keep electrodes and wire harness as immobile as possible. It may take a few minutes to stop clicking and show an ECG signal.

7.) If there is no signal or a weak signal, turn each filter (both low and high filters) to 10 Hz. Then turn the full scale" knob down to a lower value (should probably see something at 0.1).

Injectables:
1. Epinephrine (5 ug/mL if fresh (<6 mo); 10ug/mL if old (>6mo; will have pinkish color); 1mL/kg for 5 or 10 ug/kg; (bring to room temp before filling syringe)

Epi 100X Stock in H2O	**500 ug/mL**	**1000 ug/mL**
(Weigh out 5-10mg; pour into scintillation vial and add appropriate vol of H_2O. Pipette into 500mL aliquots. Store at -20°C or -80°C).	5-10mg/10-20mL	5-10mg/5-10mL
Epi Ready for Injection	**5ug/mL**	**10ug/mL**
100uL Stock + 9900 uL saline. Filter into sterile red top tube through 0.2um syringe filter. Store at 4°C, wrapped in foil. Bring to room temp before filling syringe	100uL 500ug/mL Stock + 9900 uL (9.9mL) saline.	100 uL 1000ug/mL stock + 9900 uL (9.9mL) saline

2. Vecuronium
(1mg/mL; 1mL/kg for 1mg/kg)

Reconstitute 20mg with 20mL sterile H_2O (Sicor Pharmaceuticals, Irvine, CA NDC 070302925-01)

3. HEP Saline

0.2mL of 1000IU/mL/30mL vial saline or 6.7 IU/mL (note for chronic cannulation at of AGS at UAF, we fill lines with 30 IU/mL hep saline, but are careful to not overfill cannulae and deliver high hep to animal).

4. $NaHCO_3$

NOTE at UM we always gave 0.9mL $NaHCO_3$ to rat and AGS regardless of animal weight; (1cc syringe for rats; 3 cc syringe for AGS); 8.4%; 1 mEq/mL; 3mL/kg for 3 mEq/kg) Hosira, Lake Forest, IL; NDC 0409-6625-02

5. Sterile Saline

Non injectable:

Mucomist

(1mL/500mL H_2O; 0.125 mL/1.67 mL H_2O; fill 250mL graduated cylinder with 20mL H_2O and add 40uL mucomist (acetylcysteine 10%; Roxane laboratories, Cincinnati, OH; NDC 0054-3027-02)

Arterial cannulation

Bevel slightly (best to not bevel because artery can close on bevel and give inaccurate BP reading). If artery breaks: remove silastic tubing from hemostats; rinse with saline until artery clamps and stops bleeding. Grab end with hemostats. Slide tied loop of suture over hemostat and tie off onto artery. Open other leg and cannulate other artery. It is ok to have vein and artery on opposite sides.

Resuscitation

If epi solution is pink, make fresh or animal will not resuscitate.

Appendix E

Neurological Deficit Scores (NDS) Forms

Neurological Scores:
A neurological deficit score (NDS) will be performed daily for 7 days after global cerebral ischemia. The total NDS consists of five components: consciousness and respiration, cranial nerve function, motor function, sensory function and coordination (leg/tail movement, cleaning, depth perception, righting reflex) and for motor and sensory function as previously described [Katz et al. J Cereb Blood Flow Metab, 1995, 15, 1032-1039]. The NDS ranges from 0 (normal) to 100 (brain dead).

Page 1

Animal ND score

Date Started On:_____

Animal # **Group/condition**

No			Condition	Score Guideline	Score obtained by animal each day (12:00 PM to 2:00 PM)						
					*2 hr	1	2	3	4	5	6
1	General behavioral deficit (worst, 40 points)	Consciousness	Attempt to explore spontaneously	0							
			No attempt to explore spontaneously (comatose)	20							
		Respiration	Normal	0							
			Abnormal	20							
2	Cranial nerve reflexes deficit (worst, 20 points)	Olfactory (sniffing food)	Present	0							
			Absent	5							
		Vision (follows hand)	Present	0							
			Absent	5							
		Corneal	Present	0							
			Absent	5							
		Whisker movement	Present	0							
			Absent	5							
3	Motor deficit (worst, 10 points)	Legs/tail movement	Normal	0							
			Stiff	5							
			Paralyzed	10							
4	Sensory deficit (worst, 10 points)	Legs/tail (on pinching)	Present	0							
			Absent	10							
5	Coordination deficit (worst, 20 points)	Righting reflex (attempting to right self when placed on back)	Present	0							
			Absent	10							
		Self-feeding	Present	0							
			Absent	5							
		Self-cleaning (grooming)	Present	0							
			Absent	5							
			Total	0-100							

* Denotes NDS taken 2 hours after resuscitation.

Figure E-1. "Neurological Deficit Scores (NDS)" form page 1. This form assesses neurological severity 2hr and 7-22 days post cardiac arrest injury.

Neurological Scores:
A neurological deficit score (NDS) will be performed daily for 7 days (or up to 22 days if a long-term study) after global cerebral ischemia. The total NDS consists of five components: consciousness and respiration, cranial nerve function, motor function, sensory function and coordination (leg/tail movement, cleaning, depth perception, righting reflex) and for motor and sensory function as previously described [Katz et al. J Cereb Blood Flow Metab, 1995, 15, 1032-1039]. The NDS range is a scale between 0 (normal function) to 100 (brain dead).

Page 2

Animal ND score

Date Started On:_____

Animal # **Group/condition**

No			Condition	Score Guideline	Score obtained by animal each day (12:00 PM to 2:00 PM)					
					7	10	13	16	19	22
1	General behavioral deficit (worst, 40 points)	Consciousness	Attempt to explore spontaneously	0						
			No attempt to explore spontaneously (comatose)	20						
		Respiration	Normal	0						
			Abnormal	20						
2	Cranial nerve reflexes deficit (worst, 20 points)	Olfactory (sniffing food)	Present	0						
			Absent	5						
		Vision (follows hand)	Present	0						
			Absent	5						
		Corneal	Present	0						
			Absent	5						
		Whisker movement	Present	0						
			Absent	5						
3	Motor deficit (worst, 10 points)	Legs/tail movement	Normal	0						
			Stiff	5						
			Paralyzed	10						
4	Sensory deficit (worst, 10 points)	Legs/tail (on pinching)	Present	0						
			Absent	10						
5	Coordination deficit (worst, 20 points)	Righting reflex (attempting to right self when placed on back)	Present	0						
			Absent	10						
		Self-feeding	Present	0						
			Absent	5						
		Self-cleaning (grooming)	Present	0						
			Absent	5						
			Total	0-100						

* Denotes NDS taken 2 hours after resuscitation.

Figure E-2. "Neurological Deficit Scores (NDS)" form page 2. This form assesses neurological severity 2hr and 7-22 days post cardiac arrest injury.

Appendix F

Animal Post-operative Care Log

Animal Post-Operative Care Log

Animal ID #	Check time	Date	Weight (g)	Body temp (°C)	Ambient temp (°C)	Feeding G=Gavage S=Self	Comments/Initials

Figure F-1. "Animal Post-operative Care Log" form shown. This form is used to monitor rat behavior (including any pathological changes) and recovery after cardiac arrest surgery.

Appendix G

Rat Brain Perfusion Protocol

Rat Intracardial Perfusion Procedure

Making Perfusion Solutions:

- **Filtered Saline:**
  - Dissolve 9g NaCl into 1000mL of fresh Milli-Q water and filter solution using filter paper, vacuum tubing, and a glass funnel.
  - Each animal needs 1000mL saline (prepare 1000mL but may only use about 500-600mL).
- **1000 mL of FAM**, mix following solvents:
  - 100 mL 36 % formaldehyde
  - 100 mL acetic acid, glacial (99.5 %)
  - 800 mL methanol (99.8%)

Anesthetize Rat (use isoflurane vaporizer near hood):

- Induce at 5% halothane or isoflurane (at 1.5LO2/min) for ≈ 2 min. Make sure rat is surgically anesthetized (does not respond to multiple toe pinches).
- Maintain on nose cone between 2-3% for rats.
- Proceed with the perfusion.

Position animal for collection of perfusion fluid:

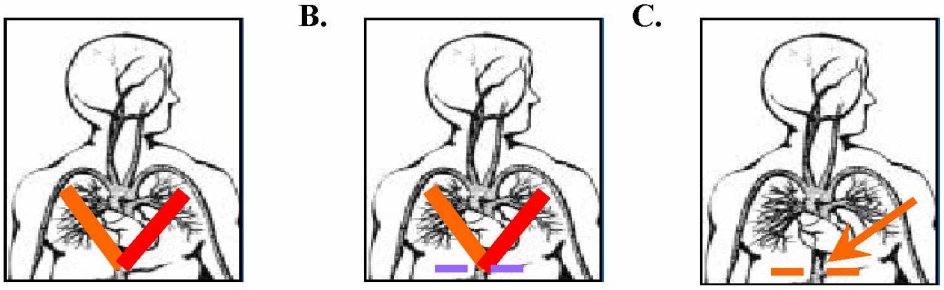
- Set animal on plastic or wire grid above 9x13 inch pan or larger.
- Fluid will run into pan
- When finished pour fluid into waste jar. Label with contents (biohazard, rat blood and FAM, [100mL 36% formaldehyde, 100mL 99.5% acetic acid glacial, & 800 mL 99.8% methanol]) and call risk management for pick up.

Perfusion Technique:

1.) Use blunt-end surgical scissors to cut a "V" in the squirrel's chest. Begin by using forceps to hold the ***xiphoid process*** of the sternum which is at the point of the "V". (**Note: the sternum is a flat, dagger shaped bone located in the middle of the**

chest. The *xiphoid process*, also called the "tip", is located on the bottom of the sternum. It is often cartilaginous, but does become bony in later years.)

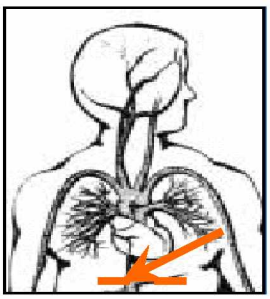
A. **B.** **C.**



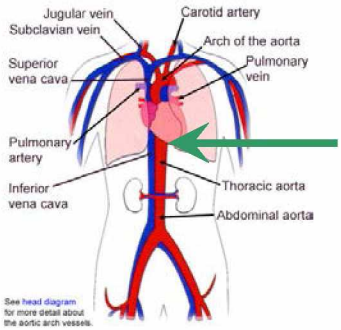
2.) Carefully cut a hole in the chest below the xiphoid process with the **surgical scissors** being careful not to hit any organs. Poke the lower blade of the scissors through the hole to cut the sides of the "V" in the animal's chest through the ribs and diaphragm. Use **large forceps** to hold the flap back.

3.) Using a **hemostat** clamp off the descending aorta. To do this you will have to push the organs aside and look for a large artery attached to the side of the body cavity near the spine. This artery is the ***descending aorta*** (directly superior to the *thoracic aorta* and inferior to the apex of the ventricle of the heart).

D.

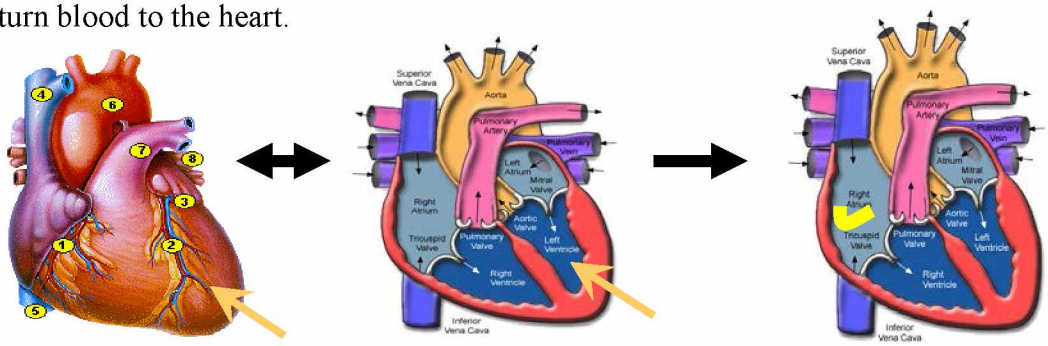


E.



4.) As shown in the above diagram (**d**), clamping the descending aorta at the green arrow (**e**) diverts the perfusion solution to only go to the upper body (e.g.. brain). The red lines are the arteries which bring blood to the body, the blue are veins that return blood to the heart.

F.



7.) (*Optional*) Insert the 18ga needle attached to the perfusion tubing into the left ventricle (**f,** yellow arrow). Be careful not to insert the needle too deep and puncture the left ventricle (**f**, yellow curve). If you can see the needle tip it is not in the heart it is only in the pericardial sac. You must be sure it goes through the heart's muscle wall but not so far as to puncture the right ventricle. After the needle is inserted into the left ventricle, cut a small hole (yellow line) in the right atrium with iris scissors to let the blood out. Then turn on the pump to begin perfusing. The perfusion solution will enter through the left ventricle go to the upper body via the ascending aorta and push the blood out via the hole in the right atrium. After a couple of minutes, you will begin to see solution exiting the body via the hole in the right atrium.

8.) Perfusion of Solutions

- The first solution to be perfused is the **saline** which should be pumped for **1 min (set pump to "2" = ~80mL/min)**
- Then the pump is shut down and the solution end of the tubing moved to the FAM solution before the pump is turned on again.
- The **FAM** solution should **pump for 19 min with pump set to "2"**. It is normal for the rat's arms to move and stiffen as they are fixed during the first 2-3 min of the FAM fix. If this does not occur, then the fix may be a poor one (only want "alive" neurons to be fixed; if poor fix then cannot use that perfused animal for neurohistopathology analysis).
- Watch the level of FAM solution in the container because you need enough left to fill your tissue collection jar (sometimes best to fill tissue collection jar with FAM prior to fixation).

(Use an 18ga needle).
Dial setting----------Flow mL/min
1----------34
2----------79.5
3----------122
4----------160
5----------201
6----------245
7----------312
8----------335

Perfusion Pump: "MasterFlex®" L/S™ Economy Drive, "Easy-Load® II"; Model # 77200-62.
Perfusion Tubing: Cole-Palmer, "MasterFlex®" Precision Tubing, Lot # 109548, Reorder # 06429-15.

9.) Brain Collection
- Using a guillotine or a pair of large scissors cut off the animal's head.
- Use a razor blade to cut the scalp and expose the skull.
- Using **rougeurs** pull bits of bone away around the head stage if the animal has one.
  - **(Note: head should be stored in FAM at 4°C for 24hr→ Head rinsed with diH_2O, brain removed & placed in FAM at 4°C for 24hr → Brain placed in 70% ethanol until fixed and ready for paraffin embedding).**
  - Always carefully side the rougeur between the skull and brain carefully and pull up so the brain is not damaged.
- When all the bone is pulled away around the head stage, slowly pull on it straight up to remove the cannulae from the brain.
- Pull off any remaining pieces of skull that may impede removing the brain.
- Turn the head upside-down over the fixative and use a spatula to loosen the brain from the skull touching only the edges of the brain.
- The dura (outer connective tissue) and the optical nerves of the brain will need to be teased away from the skull with the spatula periodically to allow the brain to slip out.
- Using a **scalpel-type spatula**, scoop out the pituitary gland at the base of the skull and place it in the fixative.
- Discard head in biohazard bag.

10.) After perfusion
- Take care of the brain: The brain is stored in fresh FAM until paraffin embedding.
- Dispose of all fluids collected during perfusion in a waste jar. Fill out Hazardous Waste form and label waste containers. Call risk management (Haz-Mat) for pick-up (**Richard Deck at 474-5617**).
- Unused FAM can be saved for future use.

Appendix H

Histology Rat Brain Trimming Instructions

<u>You will need the following items in order to trim rat brain into 3 mm slices</u>:

- **1-2 full packs of clean/new razor blades; 3 clean/new razor blades are to be used per rat brain** (use 3 new razor blades to cut coronal brain sections/slices every time you trim—this method avoids cross contamination and ensures a sharp cut every time).
- **1 clean, rat brain matrix** (make sure that each slit on matrix = 1 mm sections)
- **1 clean petri dish lid (either top or bottom lid will work fine) and 1 dropper/plastic pipet; use of either the top lid or bottom container of petri dish does not matter** (you will place the brain matrix containing your upside-down loaded brain on top of the petri dish; the petri dish lid acts as a waste container [or "alcohol reservoir"] so that you can pour 70% ethanol onto the brain so as to avoid tissue desiccation while trimming).
- **About 500-1000 mL of 70% ethanol solution** (maybe more if trimming many brains at once).
- **1 x 250 mL beaker and 1 x 600 mL beaker to act as additional 70% EtOH storage containers.** You can use these beakers to also pour alcohol onto the brain while trimming and/or to fill up any brain cassette storage containers filled with 70% EtOH.
- **1 clean plastic forceps** (to carefully pick up brain and place it upside-down inside brain matrix and to carefully transfer brain slices into cassettes after trimming).
- **A container/rat brain jar filled about half-way with 70% ethanol** (you will place your cassettes containing freshly trimmed brain slices in this container for indefinite storage until ready for paraffin embedding).
- **1 spatula; used to adjust brain position inside matrix and for anything else.**

- **Histology tissue cassettes (2 cassettes per animal: one cassette for "HPC", hippocampus, and another cassette for "STR", striatum); each cassette should be appropriately labeled as follows: "Species & Animal ID# and specific brain region (HPC/STR)" indicated on the front part of the cassette, and "cassette number or animal ID#" indicated on the side portion of the cassette.**

Actual Trimming Process:

1) **Make sure all supplies are cleaned, labeled, and ready for trimming. Brain tissue is especially prone to desiccation, so this process should be prompt.**
2) **Carefully place the brain upside-down** (so that the ***optic chiasm***, white bands "crossed together" lies superior to ***hypothalamus; e.g.*** "facing toward you") **inside the brain matrix using plastic forceps**. Make sure to place the brain matrix (with the brain inside it) on top of a petri dish lid. This acts as a reservoir for **70% ethanol (EtOH)** which you will use to consistently lubricate the brain to avoid desiccation while trimming.
3) **There will be slight variations (e.g. size of brain, shape) between animal brains**. Try to be consistent while putting brains into the matrix (e.g.. push the frontal part of the brain towards the edge of the matrix; you may have to trim off part of the ***brain stem*** in order to ensure proper brain placement inside the matrix).

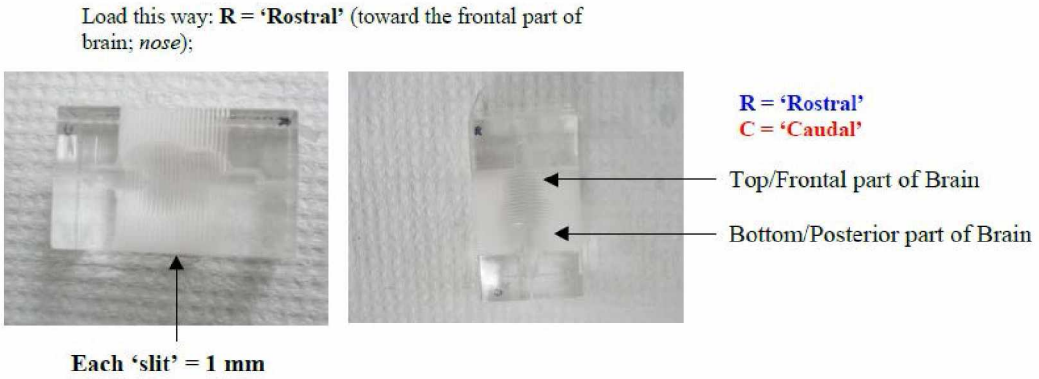


Figure 1. Shows how to load the brain inside the brain matrix.

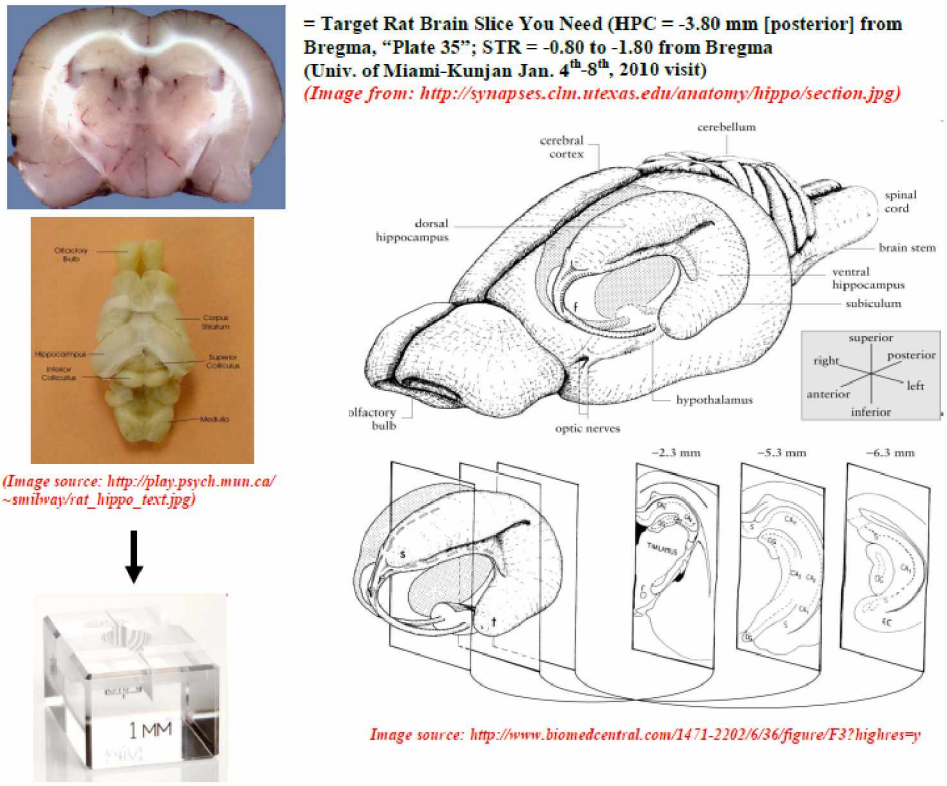


Figure 2. Shows how to load brain into brain matrix, target brain regions for slices (HPC, STR), and target slice.

4) **With the brain moisturized with 70% EtOH, place all 3 razor blades in the following order: i.) Place the 1st razor blade just under ("inferior to") the *optic chiasm* and press down slightly so that the blade is securely placed into the brain but not all the way down so as to initiate a full cut); ii.) Next, place a 2nd razor 3 mm above ("superior to") the 1st blade and press down in the same way; iii.) Lastly, place a 3rd blade 3 mm below ("inferior to") the 1st blade (middle blade) in the same way as the first two blades. The 1st blade should be a 'demarcation point' separating the *rostral* (*striatum, STR*) and *caudal* (*hippocampus, HPC*) slices from one another. Make sure that the razor blades are placed against the wall of the brain matrix slit to ensure a straight cut. Also, make sure to routinely moisturize the brain with 70% EtOH during this process to avoid desiccation.**

5) **Once all 3 razor blades have been securely placed in the brain and the brain moisturized with 70% EtOH, carefully flip the brain over to a right-side-up**

position (do this step with the brain still inside the brain matrix and containing all 3 razor blades).

6) **Gently lift up the brain matrix while carefully pressing all 3 razor blades down so as to release them from the brain matrix. Make sure to get a straight, even cut while trimming brain for slices with the brain matrix.** Crooked slices (even slightly crooked) will result in blocks that will have to be ***'matched'*** (meaning that both sides of the slice have to be retrimmed until both sides are in the same plane/region of target brain region, e.g. right and left side HPC). Uneven slices take longer to process, and thus can result in increased charges per slide.
7) **Once the brain matrix is fully off, carefully and gently transfer the HPC slice into the appropriately labeled "HPC" cassette and vice versa for the "STR" slice. When loading the HPC slice into the cassette, ALWAYS place CAUDAL SIDE DOWN** (sometimes this leads to the slice being upside down from the right-side-up position, but it shouldn't matter as long as its placed caudal side down) and **ALWAYS place the STR slice ROSTRAL side up in the appropriately labeled "STR" cassette.** You should have two slices per brain.
8) **Immediately place cassettes with HPC/STR brain slices into a container filled with 70% EtOH so as to avoid desiccation and preserve tissue integrity. Store cassettes** (containing both the HPC and STR brain slices and properly labeled with pencil) **in a container** (e.g. the same FAM Head containers work great for storing multiple cassettes too) **filled with 70% EtOH indefinitely until ready for paraffin embedding.** Based off of previous experience, **5-min hematoxylin staining** and **1-min eosin staining times** will be used. Brain slices sectioned from blocks take a total of **22-hr cycle** to set ('dry') on a microscope slide after they have been trimmed into **7-8 µm "serial" sections/slices** with the micro-vibratome (this process is done by ***Rhonda Swor, 474-6054*** **from UAF Vet Services**).

9) **<u>UAF Vet Services Costs for Histology Embedding/Slide Prep</u>**:

"Serial" sections (**unstained**, general tissue sections) = **$3 per slide.**

"Hematoxylin/Eosin" rat brain staining (or similar demanding work) = **$13 per slide**.

***For normal easy to section tissues H&E stain is $12/slide.**

***For hard to section tissues H&E stain is $14/slide.**

(Sometimes discounts are given if specific supplies are provided, e.g. ***clearing agent*** and ***alcohol*** for processing.) ****Costs subject to change annually.***

Appendix I

Histology Staining Protocol

H & E Staining Protocol for Histology Slides Bird Room 109B

A. Prepare staining racks, replenishing any low or discolored alcohol baths. Levels may be just above where tissues located on slides.
B. Filter hematoxylin.
C. First stain bath is in lower left hand corner. Proceed from left to right along front rack of baths, then move back to upper right bath & proceed from right to left along back rack of baths.
D. After staining 100 – 200 slides, change all baths. Xylenes must be put in discard bottle. Alcohols, eosin & hematoxylin must be put into waste containers as well. All wastes are picked up from EH&S.

<u>3-4 minutes Xylene bath for a total of 10 minutes</u>
1. Clear rite or Xylene bath
2. Clear rite or Xylene bath
3. Clear rite or Xylene bath

<u>30 seconds or 20-30 dips for each of the following alcohol baths</u>
4. 100% isopropyl alcohol bath
5. 100% isopropyl alcohol bath
6. 100% isopropyl alcohol bath
7. 95% isopropyl alcohol bath
8. 80% isopropyl alcohol bath
9. 70% isopropyl alcohol bath

<u>Total 1 minute gently running distilled water bath (black sink setup)</u>
10. Distilled water
11. Distilled water

12. 5 minutes in filtered Hematoxylin

Total 5 minutes gently running distilled water bath (in hood) (Hematoxylin will continue to stain even in water, so watch this time very carefully)

<u>30 Seconds or 20-30 dips for the following alcohol bath</u>
13. 80% isopropyl alcohol bath
14. Total 1 minute in Eosin, **blot rack after pulling from stain bath**

30 seconds or 20-30 dips for ***each*** of the following alcohol baths

15. 95% isopropyl alcohol bath
16. 95% isopropyl alcohol bath
17. 100% isopropyl alcohol bath
18. 100% isopropyl alcohol bath
19. 100% isopropyl alcohol bath
20. 100% isopropyl alcohol bath

30 Seconds or 20-30 dips for each of the following Xylene baths

21. Xylene bath
22. Xylene bath
23. Xylene bath

24. Coverslip (Use Richard-Allan medium with Clear rite or Cytoseal with Xylene)

Appendix J

Cardiac Arrest Supply List

Table J-1. Cardiac Arrest Supply List

Vendor/Supplier	QTY	Description	Catalogue/ Reorder No.	Price
ABAXIS, INC.	1	I-STAT CG8+ FINAL PACK (25 ct)	600-9001-25	$374.50
Accurate Surgical & Scientific Instruments	2	Fine tip, smooth inner surface 8" forcepts	FRC15RM8	$286.00
AliMed, Inc.	2	SILK SUTURE SPOOL, 100 YD, 3-0	98SUT24-4	$13.75
AliMed, Inc.	2	3-0 BLACK SILK SUTURE SPOOL, 100YD.	98SUT24-4	$27.50
American Power Conversion Corp	1	APC Replacement Battery Cartridge #2 (for UPC Upgrade Selector Surge Protector/Battery Unit)	RBC2	$39.99
ApogeeKits	1	Magnifying Glasses w/LED Light MA-016	MA-016	$18.95
Astro-Med	1	12"/Electrode/Subdermal/Platinum Needle Electrodes	F-E2-12	$214.00
BD	1	14 G x 1.75 in. BD Insyte™ Autoguard™ shielded IV catheter (2.1 mm x 45 mm) made of BD Vialon™ biomaterial. (200/ca)	381467	$507.00
Bel-Art Products	1	Gas Washing Bottle W/Fritware® Dispersion Disc	110300000	$42.00
Bickford	20	Omnicon f/air	80120	$155.00
Bickford	20	Omnicon f/air	80120	$155.00
BioMedic Data Systems	1	Implantable Programmable Temperature Transponders	IPTT-300	$995.00
BMDS	1	IPTT-300 Temperature Transponder	IPTT-300	$875.00
Braintree Scientific	1	Acrylic (Rat Brain) Matrice, 1mm Coronal	BS-A 6000C	$190.00
Braintree Scientific	1	Large Rodent Intubation Stand (400-500g)	RIS-200	$255.00
Braintree Scientific	1	Large Rat Kit for Rats 200+ grams	LR-Kit	$320.00
Cascade	1	Lidocaine HCl Injectable		$60.00
Cole Parmer	1	EW-47355-24 Cart with outlet strip and drawer, two shelf, 8" x 3" casters	47355-24	$679.00
Cole-Parmer	1	Masterflex Tygon LFL tubing L/S 15ft	EW-06429-15	$90.00
DRE Medical	1	AIR-SHIELD C-100 QT Incubator	N/A	$1,750.00
DRE Medical	N/A	AIR-SHIELD C-100 QT Incubator Shipping fee	N/A	$560.00
eBulb, Inc.	10	USHIO FCR 100W 12V Bipin Halogen Lamp (GY6.35 Bipin); Rated Life 50hr; for Nikon 80i Histology Microscope Lamphouse Unit	1000490-M	$30.02
Erlab. Inc	1	Captair Filters (2 each)	F4F	$1,130.00
Erlab. Inc	1	Prefilters (2 each)	F4PF1	$48.00
eSutures	2	3-0 Silk 8 x 18" SH taper, CR/8	C013	$14.00
eSutures	86	3-0 Surgipro blue 18" DX-19 cutting (3/8 circle, 19mm)	XP8632	$2.00

Table J-1 Continued…

Vendor/Supplier	QTY	Description	Catalogue/ Reorder No.	Price
eSutures	25	3-0 Surgipro blue 18" P-12 cutting (Prolene 8687) (expires 01/2010 or later)	SP5687	$2.00
Fine Science Tools	1	Dumont #5/45 forcepts - Dumoxel Standard Tip	11251-35	$35.50
Fine Science Tools	1	Vannas Spring Scissors-3mm blades straight	15000-00	$312.00
Fine Science Tools	2	Micro-mosquito hemostat curved 12cm	13011-12	$136.00
Fine Science Tools	1	Iris scissors delicate straight 10.5cm	14060-10	$61.75
Fine Science Tools	1	Olsen-Hegar needle holder 14cm	12002-14	$96.75
Fine Science Tools	2	Adson-brown forcepts shark teeth 12cm	11627-12	$114.00
Fine Science Tools	1	Graefe forcepts 0.8mm tips straight	11050-10	$48.25
Fine Science Tools	1	Dumont #5/45 Forcepts - Dumoxel Standard Tip	11251-35	$35.50
Fine Science Tools	1	Vannas Spring Scissors-3mm Blades Straight	15000-00	$312.00
Fine Science Tools	1	Iris Scissors - Delicate Straight 10.5cm	14060-10	$64.25
Fisher	1	Acetic Acid, Glacial, 2.5 L	RABA001025D	$71.95
Fisher	4	Reusable Polydome w/out Diaphragm	NC9554766	$22.00
Fisher	1	Black Permanent Markers	23-400-451	$30.36
Fisher	1	Red Permanent Markers	02-681-440	$30.61
Fisher	1	10uL Micro Tips	02-707-134	$353.50
Fisher	1	200uL Large Orafice Tips	21-381-8A	$317.50
Fisher	1	200uL Low Retention Tips	22-282-202	$642.50
Fisher	1	1mL Tip	13-676-10K	$80.50
Fisher	1	25mL pipettes (individual)	SCGPU05RE	$205.11
Fisher	1	500mL Stericup Filter Units	SCGPU10RE	$138.00
Fisher	1	1000mL Stericup Filter Units		$194.00
Fisher Science Education	1	Simport* MultiRack* Tube Racks 18 x 50mL centrifuge tubes (25-30mm diameter); Autoclavable, Orange, Acetal Polymer, & Cap 18; Case of 10	05-407-113; Simport Plastics; No.:S600-30O	$179.54
Fisher Scientific	1	XDCR BD P23XL BP TRANSDUCER	NC9789695	$2,000.00
Fisher Scientific	1	3.5 VOLT NUMATIC PNEU OTOSCOPE	NC9200700	$245.70
Fisher Scientific	1	Welch Allyn Rechargeable Handle	NC9085106	$214.20
Fisher Scientific	1	WELCH ALLYN BATTERIES # 72300	NC9555019	$56.04
Grass	1	Female SAFELEAD to Binding Post Adaptor	DF-215-BP	$55.00
Hallowell EMC	1	Incisor Loops (Pack of 5)	210A3490A	$30.31
Hallowell EMC	1	Lidocaine Applicator	200A3590	$24.26
Hallowell EMC	1	Rat ET Tube Introducer	210A3492	$48.51

Table J-1 Continued…

Vendor/Supplier	QTY	Description	Catalogue/ Reorder No.	Price
Hallowell EMC	1	Rat Specula, Autoclaveable	200A3588	$38.59
Hallowell EMC	1	Rodent, Tilting Work Stand	000A3467	$484.21
Hallowell EMC	1	Mouse Lidocaine Applicator	210A3496	$25.47
Hallowell EMC	1	Rat Specula, Autoclaveable	200A3588	$39.75
Hallowell EMC	1	Rodent, Tilting Work Stand	000A3467	$532.63
Harvard Apparatus	1	IMS Fluosorber Filter Canister	340415	$244.00
Harvard Apparatus	1	Christolube	Prod. # 5000047	$17.00
Harvard Apparatus	1	Fluovac 240 VAC, 50Hz	340387	$2,215.00
Henry Schein	1	Welch-Allyn Otoscope Pneumatic w/specula, 3.5V (20200)	5662532	$186.99
Henry Schein	1	Welch-Allyn Handle Rechargeable, 3.5V (71000-A)	5662474	$154.49
Henry Schein	1	Welch-Allyn Battery Rechargeable Orange, 3.5V (72300)	5662828	$45.79
Heska	1	iSTAT Cartridges 25 per box	CAT 5110-CG8+	$368.50
Heska	2	iSTAT Cartridges 25 per box	CAT 5110-CG8+	$727.00
Heska	1	iSTAT Cartridges 25 per box	CAT 5110-CG8+	$363.50
Heska	2	CG8+ iSTAT Cartridges (25 per pack)	CAT 5110-CG8+	$727.00
Heska	2	CG8+ iSTAT Cartridges (25 per pack)	CAT 5110-CG8+	$727.00
Heska	1	CG4+ iSTAT Cartridges (25 per pack)	5110-CG4+	$359.00
Heska	2	iSTAT CG4+ 25pack of cartridges	5110-CG4+	$732.00
Heska	1	CG8+ iSTAT Cartridges (25 per pack)	CAT 5110 - CG8+	$363.50
Heska	2	iSTAT CG4+ 25pack of cartridges	5110-CG4+	$359.00
Heska	2	CG8+ iSTAT Cartridges (25 per pack)	CAT 5110 - CG8+	$363.50
Heska	1	CG8+ iSTAT Cartridges (10 per pack)	CAT 5140-CG8	$162.00
Heska	1	Control solution	CAT 5130	$9.50
Heska	1	CG8+ iSTAT Cartridges (10 per pack)	CAT 5140-CG8	$162.00
Heska	1	Control solution	CAT 5130	$9.50
Heska	3	CG8+ iSTAT Cartridges (10 per pack)	CAT 5140-CG8	$486.00
Howard Electronic Instruments, Inc	1	Flushing Cannulae		$62.35
Howard Electronic Instruments, Inc.	1	JG26-0.5 Jensen Global Industrial Stainless Steel Needles, pack of 1000	JG26-0.5	$75.00
Indigo Instruments	1	Forceps, Roch Pean, 10" curved	22473	$11.00

Table J-1 Continued…

Vendor/Supplier	QTY	Description	Catalogue/ Reorder No.	Price
Indigo Instruments	1	Forceps, Roch Pean, 12" straight	22474	$13.00
Kent Scientific	1	Reusable Warming Pad, 30.5cmW X 38.1cmL (12" X 15")	TPZ-1215EA	$45.00
Kent Scientific	1	TPZ Connector for TP-500 pump		$0.00
Kent Scientific	2	TPZ connector		$0.00
Lowe's	1	Cabinet for Nikon Microscope Supplies		$51.96
Med-Vet International	1	Sharps Containers 10 gallons	SHARP-10G	$12.99
Med-Vet International	3	Sharps Containers 3 gal	SHARP-3G	$17.85
Omega	2	Hypodermic Needle Probe (30 gauge)	HYP1-30-1/2-T-G-60-SMP-M	$204.00
Omega	2	Thermocouple probe model HYP1, Type T, thermocouple	HYP1-30-1/2-T-G-60-SMP-M	$204.00
Omega	2	Benchtop Controller, type T	CSC32T	$690.00
Omega	2	Thermocouple Probe Model HYP-1	HYP1-30-1/2-T-G-60-SMP-M	$204.00
Omega	1	5-Pack, Epoxy coated tip, Type T Thermocouple	5TC-PVC-T-24-180	$50.00
Omega	1	Thermocouple	TC-PVC-T-24-180	$16.00
Omega	1	Thermocouple	HYP1-30-1/2-T-G-60-SMP-M	$102.00
Omega	1	Thermocouple probe model HYP1, Type T, thermocouple	HYP1-30-1/2-T-G-60-SMP-M	$102.00
Pall Corporation	1	ACRODISC Syringe Filters 25MM HT-200 50/PK STRL	Prod. No. 4192	$144.94
Prescott's Inc	1	PS 2001 Microscope Stand	PS 2001 (alternate # PMPOSM99)	$4,000.00
Qosina	1	Tracheal Tube Components--Polysulfone Straight Connectors (15mm x 6.0mm I.D. Tubing)	31307	
Qosina	1	2-Way, 3 Port Luer Lock, Lipid Resistant, Labeled Handle Feature, Clear Stopcock (High Density Polyethylene, Polycarbonate)	99745	
Replacement Light Bulbs	4	Philips EKE 21V 150W		$33.08
Replacement Light Bulbs	2	Philips EKE 21V 150W SKU: Philips EKE 21V 150W	93638	$16.54
Roboz	1	Mixter curved, extra delicate length 5.5"	RS-7291	$113.40
Roboz	1	Semken tip width 1.6mm, length 6", 1x2 teeth	RS-5248	$45.80

Table J-1 Continued…

Vendor/Supplier	QTY	Description	Catalogue/ Reorder No.	Price
Roboz	1	Mayo-Stille curved blade length 49mm, length 5.5"	RS-6891	$65.40
Roboz	1	Micro-Dissect Forceps (serrated, full curve, 4")	RS-5137	$45.00
Roboz	1	Blunt Scissors (5.5", curved, 42 mm)	RS-6891	$60.00
Sci Notebook	8	96-page notebook seconds	1201 seconds	$56.00
Sci Notebook	4	96-page notebook	1201	$48.00
Sigma	1	D-cycloserine 1g	C6880-1G	$54.10
Sigma	1	Paraformaldehyde, prilled, 95%	441244-1KG	$22.10
Sigma	1	Epinephrine HCL, 5 grams	E4642-5g	$38.40
Simonson Labs	4/wk	Sprague-dawley Rats: male, 180-200 grams, and 45 days old at time of order; ($21.25/rat); experimental use after 2 wks (250-350g)	S/A SIMONSEN ALBINO	$85.00
StrictlyPetSupplies .com	1	Sunbeam Products Inc Oster Professional Blade Set For A-5 Clip Size 40	UPC# 34264403772	$42.78
UAF Facilities Services	2	AirGas® Nitrous Oxide Gas Tank (N_20), $25/scrip ("refill")		$25.00
UAF Facilities Services	2	Medical Grade Oxygen Gas Tank (O_2), $25/scrip ("refill")		$25.00
UAF Vet Services	1	Vetbond		$27.10
UAF Vet Services	1	Table top unit with flow meter (no vaporizer)		$565.00
UAF Vet Services	1	Ketamine		$13.72
UAF Vet Services	1	Instrucal® Concentrated Lubricating Milk (surgical tools)		$26.60
UAF Vet Services	1	Sterile Surgical Gloves		
UAF Vet Services	30-50	Surgical Clear Plastic Mouse Drapes		
UAF Vet Services	1	1/2 Liter Non-Rebreathe Bag with specialized valve (≤$10 for a 3-L Bag)		$115.00
UAF Vet Services	1	Black fitted tubing for Rebreath Bag + 1/2 liter non-rebreathe set-up (total for both bag & tubing = $150)		$150.00
UAF Vet Services	1	10mL Heparin vial; 1000 USP Units/mL; APP Pharmaceuticals, LLC; NDC 63323-540-11		
UAF Vet Services	30	10mL Vials of Vecuronium; Hospira Inc., NDC 0409-1632-01		
UAF Vet Services	2	250mL containers of Sterile Saline Solution (one bottle for heparinized-saline dilution)		
UAF Vet Services	1	250mL container of Sterile Water		
UAF Vet Services	2	Isoflurane Liquid Anesthetic		
UAF Vet Services	2	Bottle of Sodium Bicarbonate (8.4% $NaHCO_3^-$ in a 100mL)		
UAF Vet Services	2	Epinephrine HCL, Injectable Solution		
UAF Vet Services	1	18ga x 1.5" needle from BD, 100/box (used for perfusion tubing)		

Table J-1 Continued…

Vendor/Supplier	QTY	Description	Catalogue/ Reorder No.	Price
UAF Vet Services	N/A	Gas Sterilization (e.g. cannulation packs, pressure transducer, etc.)		
UAF Vet Services	15	Small or Large "Visi-Peel Pouches size 12"x15" (for gas sterilization of pressure transducer)		
UAF Vet Services	10	Converters Self-Seal Pouches size 3 1/2" x 9 (for gas sterilization of cannulae)		
UAF Vet Services	1/ea	Medical Grade (Rx) Oxygen Gas Tank tubing, fitting, & regulator attachment		$800.00
UMECO	1	SurgiPro 3-0 Blue 18" C-14 Surgical Suture	SP684G	
Vitality Medical	1	Gaymar T Pump Localized Heat Therapy System	GMRTPP522 ea	$376.82
VWR	1	(95% Ethanol-histology grade) BDH ALCOHOL REAGENT 4L POLY	BDH1156-4LP	$35.52
VWR	2	10 mL BD Plastipak* Syringes with Eccentric Tip, BD Medical (Pack of 100); VWR Cat. No. 305482	BD305482	$29.42
VWR	1	10 mL BD Plastipak* Syringes with Eccentric Tip, BD Medical (Pack of 100); VWR Cat. No. 305482	BD305482	29.42
VWR	1	36% Formaldehyde Solution; Histology Grade Solvents, EMD Chemicals; Case of 4 Poly Bottles, 4L	EM-FX0415-4	$74.46
VWR	1	ACETIC ACID GLACIAL 99.5% 2.5L	MK250144	$16.93
VWR	2	Autoclave tape, 3/4" x60 yds	58752-704	$8.76
VWR	6	Autoclave Tape, Propper; 1.9cm x 55.4m (3/4" x 60 yds.)	268005	$31.56
VWR	1	BAG BIOHAZARD DISP19X23INPK200; HDPE, WR, SUPER BIOHAZARD DISPOSAL	11215-864	$180.88
VWR	1	BAG BIOHZ AC DSP 14X19 PK200; HDPE, WR, BIOHAZARD DISPOSAL	11215-862	$79.41
VWR	1	BD Vacutainer Venous Blood Collection Tubes (100)	VT6430	$19.70
VWR	1	BD Vacutainer Venous Blood Collection Tubes (Serum Determination Tubes, Silicone-Coated - BD366460)	VT6430	$19.70
VWR	1	BD Vacutainer Venous Blood Collection Tubes (Serum Determination Tubes, Silicone-Coated - BD366460)	VT6430	$19.70
VWR	1	Clamp Day Pinchcock (pk of 10)	21730-001	$7.56
VWR	1	Convertors TSM Sterility Maintenance Covers, Cardinal Health (8 x12)	54110-562	$102.48
VWR	1	Curity Gauze Sponges, cs of 5000	82004-740	$73.24
VWR	1	CURITY* Nonsterile Gauze Sponges, 8-Ply, Covidien; Case of 5000	82004-740	$73.24
VWR	1	CURITY* Nonsterile Gauze Sponges, 8-Ply, Covidien; Case of 5000	82004-740	73.24
VWR	1	DuPont* Mask with Earloops	80076-670	$181.12
VWR	1	GAUZE DERMACEA 4X4 8 PLY EA200	89004-984	$73.97
VWR	1	GAUZE DERMACEA 4X4 8 PLY EA200	89004-984	$73.97

Table J-1 Continued…

Vendor/Supplier	QTY	Description	Catalogue/ Reorder No.	Price
VWR	2	MAXYMum Recovery* Research-Grade Pipet Tips, Axygen Scientific (200ul - pk of 1000)	22234-070	$89.68
VWR	1	METHANOL OMNISOLV 4L	EM-MX0488-1	$67.20
VWR	1	Methanol, ACS Grade (case of 4)	BDH1135-4LG	$169.20
VWR	4	Methanol, ACS Grade, 99.8% min. (by GC); Case of 4; 4L Glass Bottles	BDH1135-4LG	$335.00
VWR	1	Methanol, ACS Grade, 99.8% min. (by GC); Case of 4; 4L Glass Bottles	BDH1135-4LG	$83.75
VWR	1	Methanol, Case of 4; 4L	BDH1135-4LG	$83.75
VWR	1	Micro Slide Holders (Pack of 12)	48457-107	$9.55
VWR	3	Needles, BD Med (Reg Bevel); Pk 100; 21ga, 25.4mm (1in.); Green	BD-305165	$36.21
VWR	5	Needles, BD Med (Reg Bevel); Pk 100; 23ga, 25.4mm (1in.); Turquoise	BD305145	$60.35
VWR	4	Needles, BD Med (Reg Bevel); Pk 100; 26ga, 12.7mm (1/2in.) ; Tan	BD305111	$48.28
VWR	1	Puritan Applicator Cotton Tip Cleaning Sticks PK 1000 (6 in. or 15 cm); Polystyrene Shafts	10805-148	$125.66
VWR	1	Puritan* Swab, Mini Cotton Head, Puritan Medical Products (100/bag, 10 bags per pack, 10pk/cs)	82004-050	$103.01
VWR	2	Single-Use Needles, BD Medical (Regular Bevel); Pk of 100; 23ga, 25.4mm (1in.); Turquoise	BD305145	$12.07
VWR	2	Single-Use Needles, BD Medical (Regular Bevel); Pk of 100; 23ga, 25.4mm (1in.); Turquoise	BD305145	12.07
VWR	1	Steel VWR® Micro Slide Double Drawer Cabinet; Stores 200 micro slide holders (up to 800 slides)	89030-198	$72.53
VWR	4	SYRINGE TUBERCULIN 1ML PK100	BD309602	$77.16
VWR	3	SYRINGE TUBERCULIN 1ML PK100	BD309602	$57.87
VWR	2	SYRINGE TUBERCULIN 1ML PK100	BD309602	$19.29
VWR	3	SYRINGE TUBERCULIN 1ML PK100	BD309602	$57.87
VWR	2	Tape Durapore 1in X 1 YD (Box of 12)--Surgical tape	56222-186	$11.44
VWR	2	Tape Durapore 1in X 1 YD (Box of 12)--Surgical tape	56222-186	11.44
VWR	1	Tape Durapore 1in X 1YD (box of 12)	56222-186	$11.44
VWR	1	Tape Durapore 1in. X 10yd BX12	56222-186	$12.25
VWR	1	Three-way stopcock	KT420163-4503	$42.22
VWR	1	Tubing LFL 3/16 x 5/16 25'	63008-866	$84.32
VWR	1	Tygon* Lab Tubing, R-3603, 3/8"IDx1/2"OD	63009-178	$25.79
VWR	2	VERSI-DRY* Lab Table Soakers, NALGENE (20" x 300')	52857-106	$281.82
VWR	1	VWR Absorbent Bench Underpads (23x24, 410mL absorbency, deluxe pads)	56616-032	$95.11
VWR	1	VWR CLOCK/HUMIDITY MONITOR (for CA Incubator)	62344-734	31.65
VWR	1	VWR Glass Jars, Wide M (500ml, cap)	89043-274	$25.57

Table J-1 Continued…

Vendor/Supplier	QTY	Description	Catalogue/ Reorder No.	Price
VWR	1	VWR PETRI DISH 100X15MM CS500	25384-070	$50.33
VWR	1	VWR Power-Free Nitrile Examination Gloves – Large (Case of 10)	82026-428	$69.58
VWR	1	VWR Power-Free Nitrile Examination Gloves – Small (Case of 10)	82026-424	$69.58
VWR	1	VWR Power-Free Nitrile Gloves - Sm (cs of 10)	82026-424	$69.58
VWR	1	VWR razor blades, pk of 100	55411-050	$25.30
VWR	1	VWR razor blades, pk of 100	55411-050	$25.30
VWR	1	VWR UNDERPAD DISP 23X24 CS200	56616-032	$42.80
VWR	1	VWR UNDERPAD DISP 23X24 CS200	56616-032	$42.80
VWR	1	VWR® Clear Glass Jars, Wide Mouth; 500ml (16oz.); Case of 12; 91 x 95mm dia. x height; Pulp/Vinyl	89043-274	$25.57
VWR	1	VWR® Powder-Free Nitrile Exam Gloves Medium	82026-426	$71.62
VWR	1	VWR® Powder-Free Nitrile Exam Gloves Medium	82026-426	71.62
VWR	1	VWR® Powder-Free Nitrile Exam Gloves Small	82026-424	$71.62
VWR	4	VWR® Razor Blades: 100 pk, Single-edge industrial razor blades, hard carbon steel, & individually wrapped	55411-050	$45.52
World Precision Instruments	1	Surgical Needles, Cutting, 5/16 circle, 18mm, size 2, pk of 12	501960	$20.00
World Precision Instruments	1	Surgical Needles, Cutting, 3/8 circle, 21mm, size 0, pk of 12	501803	$20.00
World Precision Instr.	1	Surgical Needles, Cutting, 1/2 circle, 32mm, size 5, pk of 12	501796	$20.00

Appendix K

IACUC Approval Letters for Research Protocols

UAF
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Institutional Animal Care and Use Committee
909 N Koyukuk Dr. Suite 212, P.O. Box 757270, Fairbanks, Alaska 99775-7270

November 23, 2009

To: Kelly Drew
Principal Investigator

From: University of Alaska Fairbanks IACUC

Re: [142656-3] D-cycloserine (DCS) research protocol

The IACUC reviewed and approved the Response/Follow-Up referenced below by Designated Member Review.

Received:	November 11, 2009
Approval Date:	November 23, 2009
Initial Approval Date:	November 23, 2009
Expiration Date:	November 23, 2010

Note: The revisions have answered the questions made by the committee. The protocol is approved, however approval of Standard Operating Procedures for Surgical Supplements referenced in this protocol are pending review and approval by Dr. Blake.

This action is included on the December 3, 2009 IACUC Agenda.

The PI is responsible for acquiring and maintaining all necessary permits and permissions prior to beginning work on this protocol. Failure to obtain or maintain valid permits is considered a violation of an IACUC protocol, and could result in revocation of IACUC approval.

Figure K-1. IACUC approval letter for "D-cycloserine (DCS)" research continuation.

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November 11, 2009

To: Kelly Drew
Principal Investigator
From: University of Alaska Fairbanks IACUC
Re: [142673-1] SSOP Cardiac Arrest

The IACUC reviewed and approved the New Project referenced below by Designated Member Review.

Received:	October 27, 2009
Approval Date:	November 11, 2009
Initial Approval Date:	November 11, 2009
Expiration Date:	November 11, 2010

This action is included on the November 2, 2009 IACUC Agenda.

The PI is responsible for acquiring and maintaining all necessary permits and permissions prior to beginning work on this protocol. Failure to obtain or maintain valid permits is considered a violation of an IACUC protocol, and could result in revocation of IACUC approval.

Figure K-2. IACUC approval for cardiac arrest surgical procedure. [SSOP: "Surgical Standard Operating Procedure"]

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November 10, 2009

To: Kelly Drew, PHD
Principal Investigator

From: University of Alaska Fairbanks IACUC

Re: [142652-2] Ischemia resistance research protocol 3y rewrite

The IACUC reviewed and approved the Revision referenced below by Designated Member Review.

Received:	November 5, 2009
Approval Date:	November 10, 2009
Initial Approval Date:	November 10, 2009
Expiration Date:	November 10, 2010

The revision has incorporated the recommendations and additional information requested by the committee. Approval of surgical supplements is pending review by Dr. Blake.

This action is included on the December 3, 2009 IACUC Agenda.

The PI is responsible for acquiring and maintaining all necessary permits and permissions prior to beginning work on this protocol. Failure to obtain or maintain valid permits is considered a violation of an IACUC protocol, and could result in revocation of IACUC approval.

Figure K-3. "Ischemia Resistance" IACUC approval letter. IACUC approval granted for a 3-year renewal for the "Ischemia Resistance" research protocol.

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Institutional Animal Care and Use Committee
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March 30, 2009

To: Kelly Drew, PhD
Principal Investigator

From: Erich H. Follmann, PhD
IACUC Chair

Re: IACUC Modification Request

On behalf of the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC) I have reviewed and approved the following request for modification:

Protocol#:	06-45
Title:	*Neuroprotection in hibernation*
Modification:	Increase the number of rats involved in the cardiac arrest procedure from 30 to 95. Total number of rats for 06-45 increases to 130. For surgical procedure, two rats are fasted in case there are problems with intubation or cannulation. If alternate rat not needed, animal is fed and used for acute slice or tissue collection at a later time point. The rat will not be used for cardiac arrest surgery because of the possible preconditioning from fast or exposure to isoflurane if intubation was attempted.
Received:	March 24, 2009
Approved:	March 30, 2009

Thank you for keeping your Assurance of Animal Care form Current.

Figure K-4. IACUC approval to increase total rat number for cardiac arrest experiments.

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Institutional Animal Care and Use Committee
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March 30, 2009

To: Kelly Drew, PhD
Principal Investigator

From: Erich H. Follmann, PhD
IACUC Chair

Re: IACUC Modification Request

On behalf of the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC) I have reviewed and approved the following request for modification:

Protocol#: 06-45

Title: *Neuroprotection in hibernation*

Modification: Although death is a possibility due to expected outcome following cardiac arrest, we remove death as an endpoint since animals are given supportive care. Animals will be euthanized if observed: 1) signs of infection to any wound that does not respond to treatment (Baytril 5mg/kg b.i.d SC back of neck); 2) any other complication unrelated to post cardiac arrest syndrome that would bias interpretation of outcome. Decisions regarding when euthanasia is appropriate will be made by the research team in consultation with Veterinary Services.

Received: March 24, 2009

Approved: March 30, 2009

Thank you for keeping your Assurance of Animal Care form Current.

Figure K-5. Euthanasia IACUC approval letter. Approval regarding the use of euthanasia in rats during recovery from cardiac arrest.

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Institutional Animal Care and Use Committee
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February 19, 2009

To: Kelly Drew, PhD
Principal Investigator

From: Erich H. Follmann, PhD
IACUC Chair

Re: IACUC Modification Request

On behalf of the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC) I have reviewed and approved the following request for modification:

Protocol#:	06-45
Title:	*Neuroprotection in hibernation*
Modification:	Following cardiac arrest and resuscitation animals will be perfused for histological assessment between 0 and 22 days post resuscitation.
Received:	February 19, 2009
Approved:	February 19, 2009

Thank you for keeping your Assurance of Animal Care form Current.

Figure K-6. IACUC approval for perfusion and histological assessment (0-22days) post resuscitation.

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Institutional Animal Care and Use Committee
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November 11, 2008

To: Kelly Drew, PhD
Principal Investigator

From: John Blake, DVM
Attending Veterinarian

Re: IACUC Continuing Review

On behalf of the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC) I have reviewed the request for renewal of the following assurance. This renewal request has been approved.

Protocol: #06-45

Title: *Neuroprotection in Hibernation*

Received: November 7, 2008

Approved: November 12, 2008

This Assurance is valid through November 8, 2009, but must be kept current with respect to new methods, techniques and personnel. This protocol will not be eligible for renewal in 2009; rather, it will need to be resubmitted for IACUC review/approval. We recommend submitting two months prior to the expiration date (9/8/09) to prevent any delay in the work covered by this assurance of animal care and use.

Thank you for keeping your IACUC Assurance up to date.

Figure K-7. "Neuroprotection in Hibernation" research IACUC approval. Approval granted to continue cardiac arrest research.

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Institutional Animal Care and Use Committee
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October 29, 2008

To: Kelly Drew, PhD
Principal Investigator

From: John Blake, DVM
Attending Veterinarian

Re: IACUC Modification Request

On behalf of the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC) I have reviewed and approved the following request for modification:

Protocol#:	06-45
Title:	*Neuroprotection in hibernation*
Modification:	Administration of D-cycloserine to rats 24 and 48h after cardiac arrest.
Received:	October 27, 2008
Approved:	October 29, 2008

Thank you for keeping your Assurance of Animal Care form Current.

Figure K-8. IACUC approval letter for D-cycloserine (DCS) administration 24 and 48hr after cardiac arrest injury.

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Institutional Animal Care and Use Committee
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October 16, 2008

To: Kelly Drew, PhD
Principal Investigator

From: Michael Castellini, PhD
Interim IACUC Chair

Re: IACUC Modification Request

On behalf of the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC) I have reviewed and approved the following request for modification:

Protocol#: 06-45

Title: *Neuroprotection in hibernation*

Modification: Animals prepared for cardiac arrest will be intubated. If intubation is not successful in 2 tries animals will be returned to their home cage, monitored for 4 h and again 24h later. If no signs of respiratory distress are noted animals will be used at a later date

Received: October 10, 2008

Approved: October 16, 2008

Thank you for keeping your Assurance of Animal Care form Current.

Figure K-9. IACUC approval letter shown regarding endotracheal intubation guidelines.

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October 13, 2008

To: Kelly Drew, PhD
Principal Investigator

From: John Blake, DVM
Attending Veterinarian

Re: IACUC Modification Request

On behalf of the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC) I have reviewed and approved the following request for modification:

Protocol#: 06-45

Title: *Neuroprotection in hibernation*

Modification: Allow time during endrotracheal tubation training for visualization of larynx. If technique is successful, induction using injectable anesthetic agents may replace induction with isoflurane via induction chamber.

Ketamine and medetomidine with atipamezole reversal: Rat induction dose is: 75 mg/kg BW IP and 0.5 mg/kg BW IP; atipamezole: 1 mg/kg BW IP, SC. Arctic ground squirrel dose will evaluated; the attending veterinarian requires starting at rat dose and modifying level of ketamine to achieve desired level of anesthesia. Protective eye ointment is placed post induction.

Animals used for training may be monitored for 4 hours and if no signs of respiratory distress are noted, returned to their home cage, monitored the following day and made available for subsequent experiments.

Received: October 9, 2008

Approved: October 13, 2008

Thank you for keeping your Assurance of Animal Care form Current.

Figure K-10. Ketamine and medetomidine IACUC approval. Approval to use ketamine and medetomidine during endotracheal intubation training.

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September 3, 2008

To: Kelly Drew, PhD
Principal Investigator

From: Michael Castellini, PhD
Interim IACUC Chair

Re: IACUC Modification Request

On behalf of the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC) I have reviewed and approved the following request for modification:

Protocol#:	06-45
Title:	*Neuroprotection in hibernation*
Modification:	To apply a drop of lidocaine gel to the tip of the endotracheal catheter in addition to applying it to the external surface of the epiglottis to reduce epiglottis spasms.
Received:	August 28, 2008
Approved:	September 3, 2008

Thank you for keeping your Assurance of Animal Care form Current.

Figure K-11. Lidocaine IACUC approval letter. Approval to use lidocaine during endotracheal intubation.

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Institutional Animal Care and Use Committee
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August 25, 2008

To: Kelly Drew, PhD
Principal Investigator

From: Erich H. Follmann, PhD
IACUC Chair

Re: IACUC Modification Request

On behalf of the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC) I have reviewed and approved the following request for modification:

Protocol#:	06-44, 06-45 and 07-42
Title:	*CNS Regulation of metabolic suppression, Neuroprotection in hibernation,* and *Blood Flow in Hibernation*
Modification:	Addition of Heather McFarland.
Received:	August 8, 2008
Approved:	August 25, 2008

Thank you for keeping your Assurance of Animal Care form Current.

Figure K-12. IACUC approval granted for Heather Crispell (formerly Heather McFarland).

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Institutional Animal Care and Use Committee
909 N Koyukuk Dr. Suite 212, P.O. Box 757270, Fairbanks, Alaska 99775-7270

August 15, 2008

To: Kelly Drew, PhD
Principal Investigator

From: Erich H. Follmann, PhD
IACUC Chair

Re: IACUC Modification Request

On behalf of the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC) I have reviewed and approved the following request for modification:

Protocol#:	06-45
Title:	*Neuroprotection in hibernation*
Modification:	If unexpected complications are encountered during an experimental session that will compromise interpretation of experimental results or lead to unnecessary pain or discomfort for the animal, it will be euthanized.
Received:	August 8, 2008
Approved:	August 15, 2008

Thank you for keeping your Assurance of Animal Care form Current.

Figure K-13. IACUC approval to perform euthanasia for unexpected complications during cardiac arrest surgery.

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August 15, 2008

To: Kelly Drew, PhD
Principal Investigator

From: Erich H. Follmann, PhD
IACUC Chair

Re: IACUC Modification Request

On behalf of the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC) I have reviewed and approved the following request for modification:

Protocol#:	06-45
Title:	*Neuroprotection in hibernation*
Modification:	Addition of two new objectives; training of personnel on both endotracheal intubation and cardiac arrest with resuscitation on rats and AGS. All sessions will be nonsurvival and conducted according to approved procedures. Animals used for training are included in the number of total animals approved for the project.
Received:	August 8, 2008
Approved:	August 15, 2008

Thank you for keeping your Assurance of Animal Care form Current.

Figure K-14. Intubation and resuscitation training IACUC approval.

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Institutional Animal Care and Use Committee
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June 12, 2008

To: Kelly Drew, PhD
Principal Investigator

From: Erich H. Follmann, PhD
IACUC Chair

Re: IACUC Modification Request

On behalf of the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC) I have reviewed and approved the following request for modification:

Protocol#:	06-44 and 06-45
Modification:	Authorization for Velva Combs to perform halothane anesthesia for perfusion/euthanasia.
Received:	June 11, 2008
Approved:	June 12, 2008

Thank you for keeping your Assurance of Animal Care form Current.

Figure K-15. IACUC approval granted for Velva Combs to perform anesthesia or euthanasia.

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Institutional Animal Care and Use Committee
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June 3, 2008

To: Kelly Drew, PhD
Principal Investigator

From: Erich H. Follmann, PhD
IACUC Chair

Re: IACUC Modification Request

On behalf of the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC) I have reviewed and approved the following request for modification:

Protocol#:	06-45
Title:	*Nueroprotection in Hibernation*
Modification:	The use of IPTT transponders to monitor AGS and rat body temperature.
Received:	May 29, 2008
Approved:	June 3, 2008

Thank you for keeping your Assurance of Animal Care form Current.

Figure K-16. IPTT temperature transponder IACUC approval.

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Institutional Animal Care and Use Committee
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April 3, 2008

To: Kelly Drew, PhD
Principal Investigator

From: Erich H. Follmann, PhD
IACUC Chair

Re: IACUC Modification Request

On behalf of the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC) I have reviewed and approved the following request for modification:

Protocol#:	06-45
Title:	*Neuroprotection in Hibernation*
Modification:	The addition of Tulasi Ram Jinka. The addition of pentobarb or isoflurane for intracardial perfusion prior to euthanizing animal. To replace halothane with isoflurane.
Received:	April 2, 2008
Approved:	April 3, 2008

Thank you for keeping your Assurance of Animal Care form Current.

Figure K-17. Personnel and isoflurane IACUC approval. Approval granted for the addition of Tulasi Jinka and isoflurane induction for intracardial perfusion experiments.